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(54) Title: CHROMOSOME-BASED PLATFORMS

(57) Abstract: Artificial chromosomes, including *Aces*, that have been engineered to contain available sites for site-specific, recombination-directed integration of DNA of interest are provided. These artificial chromosomes permit tractable, efficient, rational engineering of the chromosome for a variety of applications.

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CHROMOSOME-BASED PLATFORMS

RELATED APPLICATIONS

Benefit of priority to U.S. provisional application Serial No. 60/294,758, filed May 30, 2001, to Perkins, *et al.*, entitled "CHROMOSOME-BASED PLATFORMS" and to U.S. provisional application Serial No. 60/366,891, filed March 21, 2002, to Perkins, *et al.*, entitled "CHROMOSOME-BASED PLATFORMS" is claimed. Where permitted, the subject matter of which are herein incorporated by reference in their entirety.

This application is related to Provisional Application No. 60/294,687, filed May 30, 2001, by CARL PEREZ AND STEVEN FABIJANSKI entitled *PLANT ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING PLANT ARTIFICIAL CHROMOSOMES* and to U.S. Provisional Application No. 60/296,329, filed June 4, 2001, by CARL PEREZ AND STEVEN FABIJANSKI entitled *PLANT ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING PLANT ARTIFICIAL CHROMOSOMES*. This application also is related to U.S. Provisional Application No. 60/294,758, filed May 30, 2001, by EDWARD PERKINS *et al.*. entitled *CHROMOSOME-BASED PLATFORMS* and to U.S. Provisional Application No. 60/366,891, filed March 21, 2002, by by EDWARD PERKINS *et al.*. entitled *CHROMOSOME-BASED PLATFORMS*. This application is also related to U.S. application Serial Nos. (attorney dkt nos. 24601-419 and 419PC), filed on the same day herewith, entitled *PLANT ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS OF PREPARING PLANT ARTIFICIAL CHROMOSOMES* to Perez *et al.*.

This application is related to U.S. application Serial No. 08/695,191, filed August 7, 1996 by GYULA HADLACZKY and ALADAR SZALAY, entitled *ARTIFICIAL CHROMOSOMES, USES THEREOF AND*

METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES, now U.S. Patent No. 6,025,155. This application is also related to U.S. application Serial No. 08/682,080, filed July 15, 1996 by GYULA HADLACZKY and ALADAR SZALAY, entitled *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES*, now U.S. Patent No. 6,077,697. This application is also related U.S. application Serial No. 08/629,822, filed April 10, 1996 by GYULA HADLACZKY and ALADAR SZALAY, entitled *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES* (now abandoned), and is also related to copending U.S. application Serial No. 09/096,648, filed June 12, 1998, by GYULA HADLACZKY and ALADAR SZALAY, entitled *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES* and to U.S. application Serial No. 09/835,682, April 10, 1997 by GYULA HADLACZKY and ALADAR SZALAY, entitled *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES* (now abandoned). This application is also related to copending U.S. application Serial No. 09/724,726, filed November 28, 2000, U.S. application Serial No. 09/724,872, filed November 28, 2000, U.S. application Serial No. 09/724,693, filed November 28, 2000, U.S. application Serial No. 09/799,462, filed March 5, 2001, U.S. application Serial No. 09/836,911, filed April 17, 2001, and U.S. application Serial No. 10/125,767, filed April 17, 2002, each of which is by GYULA HADLACZKY and ALADAR SZALAY, and is entitled *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES*. This application is also related to International PCT application No. WO 97/40183. Where permitted the

subject matter of each of these provisional applications, international applications, and applications is incorporated by reference in its entirety.

FIELD OF INVENTION

Artificial chromosomes, including *ACes*, that have been engineered to contain available sites for site-specific, recombination-directed integration of DNA of interest are provided. These artificial chromosomes permit tractable, efficient, rational engineering of the chromosome.

BACKGROUND

Artificial chromosomes

A variety of artificial chromosomes for use in plants and animals, particularly higher plants and animals are available. In particular, U.S. Patent Nos. 6,025,155 and 6,077,697 provide heterochromatic artificial chromosomes designated therein as satellite artificial chromosomes (SATACs) and now designated artificial chromosome expression systems (*ACes*). These chromosomes are prepared by introducing heterologous DNA into a selected plant or animal cell under conditions that result in integration into a region of the chromosome that leads to an amplification event resulting in production of a dicentric chromosome. Subsequent treatment and growth of cells with dicentric chromosomes, including further amplifications, ultimately results in the artificial chromosomes provided therein. In order to introduce a desired heterologous gene (or a plurality of heterologous genes) into the artificial chromosome, the process is repeated introducing the desired heterologous genes and nucleic acids in the initial targeting step. This process is time consuming and tedious. Hence, more tractable and efficient methods for introducing heterologous nucleic acid molecules into artificial chromosomes, particularly *ACes*, are needed.

Therefore, it is an object herein to provide engineered artificial chromosomes that permit tractable, efficient and rational engineering of artificial chromosomes.

SUMMARY OF THE INVENTION

Provided herein are artificial chromosomes that permit tractable, efficient and rational engineering thereof. In particular, the artificial chromosomes provided herein contain one or a plurality of loci (sites) for site-specific, recombination-directed integration of DNA. Thus, provided herein are platform artificial chromosome expression systems ("platform *ACes*") containing single or multiple site-specific, recombination sites. The artificial chromosomes and *ACes* artificial chromosomes include plant and animal chromosomes. Any recombinase system that effects site-specific recombination is contemplated for use herein.

In one embodiment, chromosomes, including platform *ACes*, are provided that contain one or more lambda *att* sites designed for recombination-directed integration in the presence of lambda integrase, and that are mutated so that they do not require additional factors. Methods for preparing such chromosomes, vectors for use in the methods, and uses of the resulting chromosomes are also provided.

Platform *ACes* containing the recombination site(s) and methods for introducing heterologous nucleic acid into such sites and vectors therefor, are provided.

Also provided herein is a bacteriophage lambda (λ) integrase site-specific recombination system.

Methods using recombinase mediated recombination target gene expression vectors and/or genes for insertion thereof into platform chromosomes and the resulting chromosomes are provided.

Combinations and kits containing the combinations of vectors encoding a recombinase and integrase and primers for introduction of the

site recognized thereby are also provided. The kits optionally include instructions for performing site-directed integration or preparation of *ACes* containing such sites.

Also provided herein are mammalian and plant cells comprising the artificial chromosomes and *ACes* described herein. The cells can be nuclear donor cells, stem cells, such as a mesenchymal stem cell, a hematopoietic stem cell, an adult stem cell or an embryonic stem cell.

Also provide is a lamba-intR mutein comprising a glutamic acid to arginine change at position 174 of wild-type lambda-integrase3. Also provided are transgenic animals and methods for producing a transgenic animal, comprising introducing a *ACes* into an embryonic cell, such as a stem cell or embryo. The *ACes* can comprise heterologous nucleic acid that encodes a therapeutic product. The transgenic animal can be a fish, insect, reptile, amphibians, arachnid or mammal. In certain embodiments, the *ACes* is introduced by cell fusion, lipid-mediated transfection by a carrier system, microinjection, microcell fusion, electroporation, microprojectile bombardment or direct DNA transfer.

The platform *ACes*, including plant and animal *ACes*, such as MACs, provided herein can be introduced into cells, such as, but not limited to, animal cells, including mammalian cells, and into plant cells. Hence plant cells that contain platform MACs, animal cells that contain platform PACs and other combinations of cells and platform *ACes* are provided.

DESCRIPTION OF FIGURES

FIGURE 1 provides a diagram depicting creation of an exemplary *ACes* artificial chromosome prepared using methods detailed in U.S. Patent Nos. 6,025,155 and 6,077,697 and International PCT application No. WO 97/40183. In this exemplified embodiment, the nucleic acid is targeted to an acrocentric chromosome in an animal or plant, and the

heterologous nucleic acid includes a sequence-specific recombination site and marker genes.

FIGURE 2 provides a map of pWEPuro9K, which is a targeting vector derived from the vector pWE15 (GenBank Accession # X65279; SEQ ID No. 31). Plasmid pWE15 was modified by replacing the *Sal*I (Klenow filled)/*Sma*I neomycin resistance encoding fragment with the *Pvu*II/*Bam*HI (Klenow filled) puromycin resistance-encoding fragment (isolated from plasmid pPUR, Clontech Laboratories, Inc., Palo Alto, CA; GenBank Accession no. U07648; SEQ ID No. 30) resulting in plasmid pWEPuro. Subsequently a 9 Kb *Not*I fragment from the plasmid pFK161 (see Example 1, see, also Csonka *et al.* (2000) *Journal of Cell Science 113*:3207-32161; and SEQ ID NO: 118), containing a portion of the mouse rDNA region, was cloned into the *Not*I site of pWEPuro resulting in plasmid pWEPuro9K.

FIGURE 3 depicts construction of an *ACes* platform chromosome with a single recombination site, such as loxP sites or an *att*P or *att*B site. This platform *ACes* chromosome is an exemplary artificial chromosome with a single recombination site.

FIGURE 4 provides a map of plasmid pSV40-193attPsensePur.

FIGURE 5 depicts a method for formation of a chromosome platform with multiple recombination integration sites, such as attP sites.

FIGURE 6 sets forth the sequences of the core region of *att*P, *att*B, *att*L and *att*R (SEQ ID Nos. 33-36).

FIGURE 7 depicts insertional recombination of a vector encoding a marker gene, DsRed and an *att*B site with an artificial chromosome containing an *att*P site.

FIGURE 8 provides a map of plasmid pCXLamIntR (SEQ ID NO: 112), which includes the Lambda integrase (E174R)-encoding nucleic acid.

FIGURE 9 diagrammatically summarizes the platform technology; marker 1 permits selection of the artificial chromosomes containing the integration site; marker 2, which is promoterless in the target gene expression vector, permits selection of recombinants. Upon recombination with the platform marker 2 is expressed under the control of a promoter resident on the platform.

FIGURE 10 provides the vector map for the plasmid p18attBZEO-5'6XHS4eGFP (SEQ ID NO: 116).

FIGURE 11 provides the vector map for the plasmid p18attBZEO-3'6XHS4eGFP (SEQ ID NO: 115).

FIGURE 12 provides the vector map for the plasmid p18attBZEO-(6XHS4)2eGFP (SEQ ID NO: 110).

FIGURES 13 AND 14 depict the integration of a PCR product by site-specific recombination as set forth in Example 8.

FIGURE 15 provides the vector map for the plasmid pPACrDNA as set forth in Example 9.A.

DETAILED DESCRIPTION OF THE INVENTION

A. DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention(s) belong. All patents, patent applications, published applications and publications, Genbank sequences, websites and other published materials referred to throughout the entire disclosure herein, unless noted otherwise, are incorporated by reference in their entirety. Where reference is made to a URL or other such indentifier or address, it understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference thereto evidences the availability and public dissemination of such information.

As used herein, nucleic acid refers to single-stranded and/or double-stranded polynucleotides, such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), as well as analogs or derivatives of either RNA or DNA. Also included in the term "nucleic acid" are analogs of nucleic acids such as peptide nucleic acid (PNA), phosphorothioate DNA, and other such analogs and derivatives. When referring to probes or primers, optionally labeled, with a detectable label, such as a fluorescent or radiolabel, single-stranded molecules are contemplated. Such molecules are typically of a length such that they are statistically unique and of low copy number (typically less than 5, preferably less than 3) for probing or priming a library. Generally a probe or primer contains at least 14, 16 or 30 contiguous nucleotides of sequence complementary to or identical to a gene of interest. Probes and primers can be 10, 20, 30, 50, 100 or more nucleotides long.

As used herein, DNA is meant to include all types and sizes of DNA molecules including cDNA, plasmids and DNA including modified nucleotides and nucleotide analogs.

As used herein, nucleotides include nucleoside mono-, di-, and triphosphates. Nucleotides also include modified-nucleotides, such as, but are not limited to, phosphorothioate nucleotides and deazapurine nucleotides and other nucleotide analogs.

As used herein, heterologous or foreign DNA and RNA are used interchangeably and refer to DNA or RNA that does not occur naturally as part of the genome in which it is present or which is found in a location or locations and/or in amounts in a genome or cell that differ from that in which it occurs in nature. Heterologous nucleic acid is generally not endogenous to the cell into which it is introduced, but has been obtained from another cell or prepared synthetically. Generally, although not necessarily, such nucleic acid encodes RNA and proteins that are not

normally produced by the cell in which it is expressed. Any DNA or RNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which it is expressed is herein encompassed by heterologous DNA. Heterologous DNA and RNA may also encode RNA or proteins that mediate or alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes.

Examples of heterologous DNA include, but are not limited to, DNA that encodes a gene product or gene product(s) of interest, introduced for purposes of modification of the endogenous genes or for production of an encoded protein. For example, a heterologous or foreign gene may be isolated from a different species than that of the host genome, or alternatively, may be isolated from the host genome but operably linked to one or more regulatory regions which differ from those found in the unaltered, native gene. Other examples of heterologous DNA include, but are not limited to, DNA that encodes traceable marker proteins, such as a protein that confers traits including, but not limited to, herbicide, insect, or disease resistance; traits, including, but not limited to, oil quality or carbohydrate composition. Antibodies that are encoded by heterologous DNA may be secreted or expressed on the surface of the cell in which the heterologous DNA has been introduced.

As used herein, operative linkage or operative association, or grammatical variations thereof, of heterologous DNA to regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences refers to the relationship between such DNA and such sequences of nucleotides. For example, operative linkage of heterologous DNA to a promoter refers to the physical relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the

promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA.

In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5′ untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation (*i.e.*, start) codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, *e.g.*, Kozak (1991) *J. Biol. Chem.* *266*:19867-19870) can be inserted immediately 5′ of the start codon and may enhance expression.

As used herein, a sequence complementary to at least a portion of an RNA, with reference to antisense oligonucleotides, means a sequence having sufficient complementarity to be able to hybridize with the RNA, preferably under moderate or high stringency conditions, forming a stable duplex. The ability to hybridize depends on the degree of complementarity and the length of the antisense nucleic acid. The longer the hybridizing nucleic acid, the more base mismatches it can contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

As used herein, regulatory molecule refers to a polymer of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) or a polypeptide that is capable of enhancing or inhibiting expression of a gene.

As used herein, recognition sequences are particular sequences of nucleotides that a protein, DNA, or RNA molecule, or combinations thereof, (such as, but not limited to, a restriction endonuclease, a modification methylase and a recombinase) recognizes and binds. For example, a recognition sequence for Cre recombinase (see, *e.g.*, SEQ ID NO:58) is a 34 base pair sequence containing two 13 base pair inverted

repeats (serving as the recombinase binding sites) flanking an 8 base pair core and designated loxP (see, *e.g.*, Sauer (1994) *Current Opinion in Biotechnology 5*:521-527). Other examples of recognition sequences, include, but are not limited to, attB and attP, *att*R and *att*L and others (see, *e.g.*, SEQ ID Nos. 8, 41-56 and 72), that are recognized by the recombinase enzyme Integrase (see, SEQ ID Nos. 37 and 38 for the nucleotide and encoded amino acid sequences of an exemplary lambda phage integrase).

The recombination site designated *att*B is an approximately 33 base pair sequence containing two 9 base pair core-type Int binding sites and a 7 base pair overlap region; *att*P (SEQ ID No. 72) is an approximately 240 base pair sequence containing core-type Int binding sites and arm-type Int binding sites as well as sites for auxiliary proteins IHF, FIS, and Xis (see, *e.g.*, Landy (1993) *Current Opinion in Biotechnology 3*:699-707l see, *e.g.*, SEQ ID Nos. 8 and 72).

As used herein, a recombinase is an enzyme that catalyzes the exchange of DNA segments at specific recombination sites. An integrase herein refers to a recombinase that is a member of the lambda (λ) integrase family.

As used herein, recombination proteins include excisive proteins, integrative proteins, enzymes, co-factors and associated proteins that are involved in recombination reactions using one or more recombination sites (see, Landy (1993) *Current Opinion in Biotechnology 3*:699-707). The recombination proteins used herein can be delivered to a cell via an expression cassette on an appropriate vector, such as a plasmid, and the like. In other embodiments, the recombination proteins can be delivered to a cell in protein form in the same reaction mixture used to deliver the desired nucleic acid, such as a platform *ACes*, donor target vectors, and the like.

As used herein the expression "lox site" means a sequence of nucleotides at which the gene product of the cre gene, referred to herein as Cre, can catalyze a site-specific recombination event. A LoxP site is a 34 base pair nucleotide sequence from bacteriophage P1 (see, *e.g.*, Hoess *et al.* (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*:3398-3402). The LoxP site contains two 13 base pair inverted repeats separated by an 8 base pair spacer region as follows: (SEQ ID NO. 57):

ATAACTTCGTATA ATGTATGC TATACGAAGTTAT

*E. coli*DH5Δlac and yeast strain BSY23 transformed with plasmid pBS44 carrying two loxP sites connected with a LEU2 gene are available from the American Type Culture Collection (ATCC) under accession numbers ATCC 53254 and ATCC 20773, respectively. The lox sites can be isolated from plasmid pBS44 with restriction enzymes *Eco*RI and *Sal*I, or *Xho*I and *Bam*HI. In addition, a preselected DNA segment can be inserted into pBS44 at either the *Sal*I or *Bam*HI restriction enzyme sites. Other lox sites include, but are not limited to, LoxB, LoxL, LoxC2 and LoxR sites, which are nucleotide sequences isolated from *E. coli* (see, *e.g.*, Hoess *et al.* (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*:3398). Lox sites can also be produced by a variety of synthetic techniques (see, *e.g.*, Ito *et al.* (1982) *Nuc. Acid Res. 10:*1755 and Ogilvie *et al.* (1981) *Science 270*:270).

As used herein, the expression "cre gene" means a sequence of nucleotides that encodes a gene product that effects site-specific recombination of DNA in eukaryotic cells at lox sites. One cre gene can be isolated from bacteriophage P1 (see, *e.g.*, Abremski *et al.* (1983) *Cell 32*:1301-1311). *E. coli* DH1 and yeast strain BSY90 transformed with plasmid pBS39 carrying a cre gene isolated from bacteriophage P1 and a GAL1 regulatory nucleotide sequence are available from the American Type Culture Collection (ATCC) under accession numbers ATCC 53255

and ATCC 20772, respectively. The cre gene can be isolated from plasmid pBS39 with restriction enzymes *Xho*I and *Sal*I.

As used herein, site-specific recombination refers to site-specific recombination that is effected between two specific sites on a single nucleic acid molecule or between two different molecules that requires the presence of an exogenous protein, such as an integrase or recombinase.

For example, Cre-lox site-specific recombination can include the following three events:

a. deletion of a pre-selected DNA segment flanked by lox sites;

b. inversion of the nucleotide sequence of a pre-selected DNA segment flanked by lox sites; and

c. reciprocal exchange of DNA segments proximate to lox sites located on different DNA molecules.

This reciprocal exchange of DNA segments can result in an integration event if one or both of the DNA molecules are circular. DNA segment refers to a linear fragment of single- or double-stranded deoxyribonucleic acid (DNA), which can be derived from any source. Since the lox site is an asymmetrical nucleotide sequence, two lox sites on the same DNA molecule can have the same or opposite orientations with respect to each other. Recombination between lox sites in the same orientation results in a deletion of the DNA segment located between the two lox sites and a connection between the resulting ends of the original DNA molecule. The deleted DNA segment forms a circular molecule of DNA. The original DNA molecule and the resulting circular molecule each contain a single lox site. Recombination between lox sites in opposite orientations on the same DNA molecule result in an inversion of the nucleotide sequence of the DNA segment located between the two lox

sites. In addition, reciprocal exchange of DNA segments proximate to lox sites located on two different DNA molecules can occur. All of these recombination events are catalyzed by the gene product of the cre gene. Thus, the Cre-lox system can be used to specifically delete, invert, or insert DNA. The precise event is controlled by the orientation of lox DNA sequences, in *cis* the lox sequences direct the *Cre* recombinase to either delete (lox sequences in direct orientation) or invert (lox sequences in inverted orientation) DNA flanked by the sequences, while *in trans* the lox sequences can direct a homologous recombination event resulting in the insertion of a recombinant DNA.

As used herein, a chromosome is a nucleic acid molecule, and associated proteins, that is capable of replication and segregation within a cell upon cell division. Typically, a chromosome contains a centromeric region, replication origins, telomeric regions and a region of nucleic acid between the centromeric and telomeric regions.

As used herein, a centromere is any nucleic acid sequence that confers an ability to segregate to daughter cells through cell division. A centromere may confer stable segregation of a nucleic acid sequence, including an artificial chromosome containing the centromere, through mitotic or meiotic divisions, including through both mitotic and meiotic divisions. A particular centromere is not necessarily derived from the same species in which it is introduced, but has the ability to promote DNA segregation in cells of that species.

As used herein, euchromatin and heterochromatin have their recognized meanings. Euchromatin refers to chromatin that stains diffusely and that typically contains genes, and heterochromatin refers to chromatin that remains unusually condensed and that has been thought to be transcriptionally inactive. Highly repetitive DNA sequences (satellite DNA) are usually located in regions of the heterochromatin surrounding

the centromere (pericentric or pericentromeric heterochromatin). Constitutive heterochromatin refers to heterochromatin that contains the highly repetitive DNA which is constitutively condensed and genetically inactive.

As used herein, an acrocentric chromosome refers to a chromosome with arms of unequal length.

As used herein, endogenous chromosomes refer to genomic chromosomes as found in a cell prior to generation or introduction of an artificial chromosome.

As used herein, artificial chromosomes are nucleic acid molecules, typically DNA, that stably replicate and segregate alongside endogenous chromosomes in cells and have the capacity to accommodate and express heterologous genes contained therein. It has the capacity to act as a gene delivery vehicle by accommodating and expressing foreign genes contained therein. A mammalian artificial chromosome (MAC) refers to chromosomes that have an active mammalian centromere(s). Plant artificial chromosomes, insect artificial chromosomes and avian artificial chromosomes refer to chromosomes that include centromeres that function in plant, insect and avian cells, respectively. A human artificial chromosome (HAC) refers to chromosomes that include centromeres that function in human cells. For exemplary artificial chromosomes, see, *e.g.*, U.S. Patent Nos. 6,025,155; 6,077,697; 5,288,625; 5,712,134; 5,695,967; 5,869,294; 5,891,691 and 5,721,118 and published International PCT application Nos, WO 97/40183 and WO 98/08964. Artificial chromosomes include those that are predominantly heterochromatic (formerly referred to as satellite artificial chromosomes (SATACs); see, *e.g.*, U.S. Patent Nos. 6,077,697 and 6,025,155 and published International PCT application No. WO 97/40183), minichromosomes that contain a *de novo* centromere (see, U.S. Patent

Nos. 5,712,134, 5,891,691 and 5,288,625), artificial chromosomes predominantly made up of repeating nucleic acid units and that contain substantially equivalent amounts of euchromatic and heterochromatic DNA and *in vitro* assembled artificial chromosomes (see, copending U.S. provisional application Serial No. 60/294,687, filed on May 30, 2001).

As used herein, the term "satellite DNA-based artificial chromosome (SATAC)" is interchangable with the term "artificial chromosome expression system (*ACes*)". These artificial chromosomes (*ACes*) include those that are substantially all neutral non-coding sequences (heterochromatin) except for foreign heterologous, typically gene-encoding nucleic acid, that is interspersed within the heterochromatin for the expression therein (see U.S. Patent Nos. 6,025,155 and 6,077,697 and International PCT application No. WO 97/40183), or that is in a single locus as provided herein. Also included are *ACes* that may include euchromatin and that result from the process described in U.S. Patent Nos. 6,025,155 and 6,077,697 and International PCT application No. WO 97/40183 and outlined herein. The delineating structural feature is the presence of repeating units, that are generally predominantly heterochromatin. The precise structure of the *ACes* will depend upon the structure of the chromosome in which the initial amplification event occurs; all share the common feature of including a defined pattern of repeating units. Generally *ACes* have more heterochromatin than euchromatin. Foreign nucleic acid molecules (heterologous genes) contained in these artificial chromosome expression systems can include any nucleic acid whose expression is of interest in a particular host cell. Such foreign nucleic acid molecules, include, but are not limited to, nucleic acid that encodes traceable marker proteins (reporter genes), such as fluorescent proteins, such as green, blue or red fluorescent proteins (GFP, BFP and RFP, respectively), other reporter

genes, such as β-galactosidase and proteins that confer drug resistance, such as a gene encoding hygromycin-resistance. Other examples of heterologous nucleic acid molecules include, but are not limited to, DNA that encodes therapeutically effective substances, such as anti-cancer agents, enzymes and hormones, DNA that encodes other types of proteins, such as antibodies, and DNA that encodes RNA molecules (such as antisense or siRNA molecules) that are not translated into proteins.

As used herein, an artificial chromosome platform, also referred to herein as a "platform *ACes*" or "*ACes* platform", refers to an artificial chromosome that has been engineered to include one or more sites for site-specific, recombination-directed integration. In particular, *ACes* that are so-engineered are provided. Any sites, including but not limited to any described herein, that are suitable for such integration are contemplated. Plant and animal platform *ACes* are provided. Among the *ACes* contemplated herein are those that are predominantly heterochromatic (formerly referred to as satellite artificial chromosomes (SATACs); see, *e.g.*, U.S. Patent Nos. 6,077,697 and 6,025,155 and published International PCT application No. WO 97/40183), artificial chromosomes predominantly made up of repeating nucleic acid units and that contain substantially equivalent amounts of euchromatic and heterochromatic DNA resulting from an amplification event depicted in the referenced patent and herein. Included among the *ACes* for use in generating platforms, are artificial chromosomes that introduce and express heterologous nucleic acids in plants (see, copending U.S. provisional application Serial No. 60/294,687, filed on May 30, 2001). These include artificial chromosomes that have a centromere derived from a plant, and, also, artificial chromosomes that have centromeres that may be derived from other organisms but that function in plants.

As used herein a "reporter *ACes*" refers to a an *ACes* that comprises one or a plurality of reporter constructs, where the reporter construct comprises a reporter gene in operative linkage with a regulatory region responsive to test or known compounds.

As used herein, amplification, with reference to DNA, is a process in which segments of DNA are duplicated to yield two or multiple copies of substantially similar or identical or nearly identical DNA segments that are typically joined as substantially tandem or successive repeats or inverted repeats.

As used herein, amplification-based artificial chromosomes are artificial chromosomes derived from natural or endogenous chromosomes by virtue of an amplification event, such as one initiated by introduction of heterologous nucleic acid into rDNA in a chromosome. As a result of such an event, chromosomes and fragments thereof exhibiting segmented or repeating patterns arise. Artificial chromosomes can be formed from these chromosomes and fragments. Hence, amplification-based artificial chromosomes refer to engineered chromosomes that exhibit an ordered segmentation that is not observed in naturally occurring chromosomes and that distinguishes them from naturally occurring chromosomes. The segmentation, which can be visualized using a variety of chromosome analysis techniques known to those of skill in the art, correlates with the structure of these artificial chromosomes. In addition to containing one or more centromeres, the amplification-based artificial chromosomes, throughout the region or regions of segmentation are predominantly made up of nucleic acid units also referred to as "amplicons", that is (are) repeated in the region and that have a similar gross structure. Repeats of an amplicon tend to be of similar size and share some common nucleic acid sequences. For example, each repeat of an amplicon may contain a replication site involved in amplification of chromosome segments and/or

some heterologous nucleic acid that was utilized in the initial production of the artificial chromosome. Typically, the repeating units are substantially similar in nucleic acid composition and may be nearly identical.

The amplification-based artificial chromosomes differ depending on the chromosomal region that has undergone amplification in the process of artificial chromosome formation. The structures of the resulting chromosomes can vary depending upon the initiating event and/or the conditions under which the heterologous nucleic acid is introduced, including modification to the endogenous chromosomes. For example, in some of the artificial chromosomes provided herein, the region or regions of segmentation may be made up predominantly of heterochromatic DNA. In other artificial chromosomes provided herein, the region or regions of segmentation may be made up predominantly of euchromatic DNA or may be made up of similar amounts of heterochromatic and euchromatic DNA.

As used herein an amplicon is a repeated nucleic acid unit. In some of the artificial chromosomes described herein, an amplicon may contain a set of inverted repeats of a megareplicon. A megareplicon represents a higher order replication unit. For example, with reference to some of the predominantly heterochromatic artificial chromosomes, the megareplicon can contain a set of tandem DNA blocks (*e.g.*, ~7.5 Mb DNA blocks) each containing satellite DNA flanked by non-satellite DNA or may be made up of substantially rDNA. Contained within the megareplicon is a primary replication site, referred to as the megareplicator, which may be involved in organizing and facilitating replication of the pericentric heterochromatin and possibly the centromeres. Within the megareplicon there may be smaller (*e.g.*, 50-300 kb) secondary replicons.

In artificial chromosomes, such as those provided U.S. Patent Nos. 6,025,155 and 6,077,697 and International PCT application No. WO 97/40183, the megareplicon is defined by two tandem blocks (~7.5 Mb DNA blocks in the chromosomes provided therein). Within each artificial chromosome or among a population thereof, each amplicon has the same gross structure but may contain sequence variations. Such variations will arise as a result of movement of mobile genetic elements, deletions or insertions or mutations that arise, particularly in culture. Such variation does not affect the use of the artificial chromosomes or their overall structure as described herein.

As used herein, amplifiable, when used in reference to a chromosome, particularly the method of generating artificial chromosomes provided herein, refers to a region of a chromosome that is prone to amplification. Amplification typically occurs during replication and other cellular events involving recombination (*e.g.*, DNA repair). Such regions include regions of the chromosome that contain tandem repeats, such as satellite DNA, rDNA, and other such sequences.

As used herein, a dicentric chromosome is a chromosome that contains two centromeres. A multicentric chromosome contains more than two centromeres.

As used herein, a formerly dicentric chromosome is a chromosome that is produced when a dicentric chromosome fragments and acquires new telomeres so that two chromosomes, each having one of the centromeres, are produced. Each of the fragments is a replicable chromosome. If one of the chromosomes undergoes amplification of primarily euchromatic DNA to produce a fully functional chromosome that is predominantly (at least more than 50%) euchromatin, it is a minichromosome. The remaining chromosome is a formerly dicentric chromosome. If one of the chromosomes undergoes amplification,

whereby heterochromatin (such as, for example, satellite DNA) is amplified and a euchromatic portion (such as, for example, an arm) remains, it is referred to as a sausage chromosome. A chromosome that is substantially all heterochromatin, except for portions of heterologous DNA, is called a predominantly heterochromatic artificial chromosome. Predominantly heterochromatic artificial chromosomes can be produced from other partially heterochromatic artificial chromosomes by culturing the cell containing such chromosomes under conditions such as BrdU treatment that destabilize the chromosome and/or growth under selective conditions so that a predominantly heterochromatic artificial chromosome is produced. For purposes herein, it is understood that the artificial chromosomes may not necessarily be produced in multiple steps, but may appear after the initial introduction of the heterologous DNA. Typically, artificial chromosomes appear after about 5 to about 60, or about 5 to about 55, or about 10 to about 55 or about 25 to about 55 or about 35 to about 55 cell doublings after initiation of artificial chromosome generation, or they may appear after several cycles of growth under selective conditions and BrdU treatment.

As used herein, an artificial chromosome that is predominantly heterochromatic (*i.e.*, containing more heterochromatin than euchromatin, typically more than about 50%, more than about 70%, or more than about 90% heterochromatin) may be produced by introducing nucleic acid molecules into cells, such as, for example, animal or plant cells, and selecting cells that contain a predominantly heterochromatic artificial chromosome. Any nucleic acid may be introduced into cells in such methods of producing the artificial chromosomes. For example, the nucleic acid may contain a selectable marker and/or optionally a sequence that targets nucleic acid to the pericentric, heterochromatic region of a chromosome, such as in the short arm of acrocentric chromosomes and

nucleolar organizing regions. Targeting sequences include, but are not limited to, lambda phage DNA and rDNA for production of predominantly heterochromatic artificial chromosomes in eukaryotic cells.

After introducing the nucleic acid into cells, a cell containing a predominantly heterochromatic artificial chromosome is selected. Such cells may be identified using a variety of procedures. For example, repeating units of heterochromatic DNA of these chromosomes may be discerned by G-banding and/or fluorescence in situ hybridization (FISH) techniques. Prior to such analyses, the cells to be analyzed may be enriched with artificial chromosome-containing cells by sorting the cells on the basis of the presence of a selectable marker, such as a reporter protein, or by growing (culturing) the cells under selective conditions. It is also possible, after introduction of nucleic acids into cells, to select cells that have a multicentric, typically dicentric, chromosome, a formerly multicentric (typically dicentric) chromosome and/or various heterochromatic structures, such as a megachromosome and a sausage chromosome, that contain a centromere and are predominantly heterochromatic and to treat them such that desired artificial chromosomes are produced. Cells containing a new chromosome are selected. Conditions for generation of a desired structure include, but are not limited to, further growth under selective conditions, introduction of additional nucleic acid molecules and/or growth under selective conditions and treatment with destabilizing agents, and other such methods (see International PCT application No. WO 97/40183 and U.S. Patent Nos. 6,025,155 and 6,077,697).

As used herein, a "selectable marker" is a nucleic acid segment, generally DNA, that allows one to select for or against a molecule or a cell that contains it, often under particular conditions. These markers can encode an activity, such as, but not limited to, production of RNA,

peptide, or protein, or can provide a binding site for RNA, peptides, proteins, inorganic and organic compounds and compositions. Examples of selectable markers include but are not limited to: (1) nucleic acid segments that encode products that provide resistance against otherwise toxic compounds (e.g., antibiotics); (2) nucleic acid segments that encode products that are otherwise lacking in the recipient cell (e.g., tRNA genes, auxotrophic markers); (3) nucleic acid segments that encode products that suppress the activity of a gene product; (4) nucleic acid segments that encode products that can be identified, such as phenotypic markers, including β-galactosidase, red, blue and/or green fluorescent proteins (FPs), and cell surface proteins; (5) nucleic acid segments that bind products that are otherwise detrimental to cell survival and/or function; (6) nucleic acid segments that otherwise inhibit the activity of any of the nucleic acid segments described in Nos. 1-5 above (e.g., antisense oligonucleotides or siRNA molecules for use in RNA interference); (7) nucleic acid segments that bind products that modify a substrate (e.g. restriction endonucleases); (8) nucleic acid segments that can be used to isolate a desired molecule (e.g. specific protein binding sites); (9) nucleic acid segments that encode a specific nucleotide sequence that can be otherwise non-functional, such as for PCR amplification of subpopulations of molecules; and/or (10) nucleic acid segments, which when absent, directly or indirectly confer sensitivity to particular compounds. Thus, for example, selectable markers include nucleic acids encoding fluorescent proteins, such as green fluorescent proteins, β-galactosidase and other readily detectable proteins, such as chromogenic proteins or proteins capable of being bound by an antibody and FACs sorted. Selectable markers such as these, which are not required for cell survival and/or proliferation in the presence of a selection agent, are also referred to herein as reporter molecules. Other selectable markers, *e.g.*, the

neomycin phosphotransferase gene, provide for isolation and identification of cells containing them by conferring properties on the cells that make them resistant to an agent, *e.g.*, a drug such as an antibiotic, that inhibits proliferation of cells that do not contain the marker.

As another example, interference of gene expression by double stranded RNA has been shown in Caenorhabditis elegans, plants, Drosophila, protozoans and mammals. This method is known as RNA interference (RNAi) and utilizes short, double-stranded RNA molecules (siRNAs). The siRNAs are generally composed of a 19-22bp double-stranded RNA stem, a loop region and a 1-4 bp overhang on the 3' end. The reduction of gene expression has been accomplished by direct introduction of the siRNAs into the cell (Harborth J et al., 2001, J Cell Sci 114(pt 24):4557-65) as well as the introduction of DNA encoding and expressing the siRNA molecule. The encoded siRNA molecules are under the regulation of an RNA polymerase III promoter (see, e.g., Yu et al., 2002, Proc Natl Acad Sci USA 99(9);6047-52; Brummelkamp et al., 2002, Science 296(5567):550-3; Miyagishi et al., 2002, Nat Biotechnol 20(5):497-500; and the like). In certain embodiments, RNAi in mammalian cells may have advantages over other therapeutic methods. For example, producing siRNA molecules that block viral genetic activities in infected cells may reduce the effects of the virus. Platform *ACes* provided herein encoding siRNA molecule(s) are an additional utilization of the platform *ACes* technology. The platform *ACes* could be engineered to encode one or more siRNA molecules to create gene "knockdowns". In one embodiment, a platform *ACes* can engineered to encode both the siRNA molecule and a replacement gene. For example, a mouse model or cell culture system could be generated using a platform *ACes* that has a knockdown of the endogenous mouse gene, by siRNA, and the human gene homolog expressing in place of the mouse gene. The placement of

siRNA encoding sequences under the regulation of a regulatable or inducible promoter would allow one to temporally and/or spatially control the knockdown effect of the corresponding gene.

As used herein, a reporter gene includes any gene that expresses a detectable gene product, which may be RNA or protein. Generally reporter genes are readily detectable. Examples of reporter genes include, but are not limited to nucleic acid encoding a fluorescent protein, CAT (chloramphenicol acetyl transferase) (Alton *et al.* (1979) *Nature 282*: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet *et al.* (1987) *Mol. Cell. Biol.* *7*:725-737); bacterial luciferase (Engebrecht and Silverman (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*:4154-4158; Baldwin *et al.* (1984) *Biochemistry 23*:3663-3667); and alkaline phosphatase (Toh *et al.* (1989) *Eur. J. Biochem. 182*:231-238, Hall *et al.* (1983) *J. Mol. Appl. Gen.* *2*:101).

As used herein, growth under selective conditions means growth of a cell under conditions that require expression of a selectable marker for survival.

As used herein, an agent that destabilizes a chromosome is any agent known by those skilled in the art to enhance amplification events, and/or mutations. Such agents, which include BrdU, are well known to those skilled in the art.

In order to generate an artificial chromosome containing a particular heterologous nucleic acid of interest, it is possible to include the nucleic acid in the nucleic acid that is being introduced into cells to initiate production of the artificial chromosome. Thus, for example, a nucleic acid can be introduced into a cell along with nucleic acid encoding a selectable marker and/or a nucleic acid that targets to a heterochromatic region of a chromosome. For introducing a heterologous nucleic acid into

the cell, it can be included in a fragment that includes a selectable marker or as part of a separate nucleic acid fragment and introduced into the cell with a selectable marker during the process of generating the artificial chromosomes. Alternatively, heterologous nucleic acid can be introduced into an artificial chromosome at a later time after the initial generation of the artificial chromosome.

As used herein, the minichromosome refers to a chromosome derived from a multicentric, typically dicentric, chromosome that contains more euchromatic than heterochromatic DNA. For purposes herein, the minichromosome contains a *de novo* centromere (e.g., a neocentromere). In some embodiments, for example, the minichromosome contains a centromere that replicates in animals, e.g., a mammalian centromere or in plants, e.g., a plant centromere.

As used herein, *in vitro* assembled artificial chromosomes or synthetic chromosomes can be either more euchromatic than heterochromatic or more heterochromatic than euchromatic and are produced by joining essential components of a chromosome *in vitro*. These components include at least a centromere, a megareplicator, a telomere and optionally secondary origins of replication.

As used herein, *in vitro* assembled plant or animal artificial chromosomes are produced by joining essential components (at least the centromere, telomere(s), megareplicator and optional secondary origins of replication) that function in plants or animals. In particular embodiments, the megareplicator contains sequences of rDNA, particularly plant or animal rDNA.

As used herein, a plant is a eukaryotic organism that contains, in addition to a nucleus and mitochondria, chloroplasts capable of carrying out photosynthesis. A plant can be unicellular or multicellular and can contain multiple tissues and/or organs. Plants can reproduce sexually or

asexually and can be perennial or annual in growth. Plants can also be terrestrial or aquatic. The term "plant" includes a whole plant, plant cell, plant protoplast, plant calli, plant seed, plant organ, plant tissue, and other parts of a whole plant.

As used herein, stable maintenance of chromosomes occurs when at least about 85%, preferably 90%, more preferably 95%, of the cells retain the chromosome. Stability is measured in the presence of a selective agent. Preferably these chromosomes are also maintained in the absence of a selective agent. Stable chromosomes also retain their structure during cell culturing, suffering no unintended intrachromosomal or interchromosomal rearrangements.

As used herein, *de novo* with reference to a centromere, refers to generation of an excess centromere in a chromosome as a result of incorporation of a heterologous nucleic acid fragment using the methods herein.

As used herein, BrdU refers to 5-bromodeoxyuridine, which during replication is inserted in place of thymidine. BrdU is used as a mutagen; it also inhibits condensation of metaphase chromosomes during cell division.

As used herein, ribosomal RNA (rRNA) is the specialized RNA that forms part of the structure of a ribosome and participates in the synthesis of proteins. Ribosomal RNA is produced by transcription of genes which, in eukaryotic cells, are present in multiple copies. In human cells, the approximately 250 copies of rRNA genes (i.e., genes which encode rRNA) per haploid genome are spread out in clusters on at least five different chromosomes (chromosomes 13, 14, 15, 21 and 22). In mouse cells, the presence of ribosomal DNA (rDNA, which is DNA containing sequences that encode rRNA) has been verified on at least 11 pairs out of 20 mouse chromosomes (chromosomes 5, 6, 7, 9, 11, 12, 15, 16, 17, 18, and 19)

(see e.g., Rowe *et al.* (1996) *Mamm. Genome* 7:886-889 and Johnson *et al.* (1993) *Mamm. Genome* 4:49-52). In *Arabidopsis thaliana* the presence of rDNA has been verified on chromosomes 2 and 4 (18S, 5.8S, and 25S rDNA) and on chromosomes 3,4, and 5 (5S rDNA)(see The Arabidopsis Genome Initiative (2000) Nature 408:796-815). In eukaryotic cells, the multiple copies of the highly conserved rRNA genes are located in a tandemly arranged series of rDNA units, which are generally about 40-45 kb in length and contain a transcribed region and a nontranscribed region known as spacer (i.e., intergenic spacer) DNA which can vary in length and sequence. In the human and mouse, these tandem arrays of rDNA units are located adjacent to the pericentric satellite DNA sequences (heterochromatin). The regions of these chromosomes in which the rDNA is located are referred to as nucleolar organizing regions (NOR) which loop into the nucleolus, the site of ribosome production within the cell nucleus.

As used herein, a megachromosome refers to a chromosome that, except for introduced heterologous DNA, is substantially composed of heterochromatin. Megachromosomes are made up of an array of repeated amplicons that contain two inverted megareplicons bordered by introduced heterologous DNA (see, *e.g.*, Figure 3 of U.S. Patent No. 6,077,697 for a schematic drawing of a megachromosome). For purposes herein, a megachromosome is about 50 to 400 Mb, generally about 250-400 Mb. Shorter variants are also referred to as truncated megachromosomes (about 90 to 120 or 150 Mb), dwarf megachromosomes (~150-200 Mb), and a micro-megachromosome (~50-90 Mb, typically 50-60 Mb). For purposes herein, the term

megachromosome refers to the overall repeated structure based on an array of repeated chromosomal segments (amplicons) that contain two inverted megareplicons bordered by any inserted heterologous DNA. The size will be specified.

As used herein, gene therapy involves the transfer or insertion of nucleic acid molecules into certain cells, which are also referred to as target cells, to produce specific products that are involved in preventing, curing, correcting, controlling or modulating diseases, disorders and deleterious conditions. The nucleic acid is introduced into the selected target cells in a manner such that the nucleic acid is expressed and a product encoded thereby is produced. Alternatively, the nucleic acid may in some manner mediate expression of DNA that encodes a therapeutic product. This product may be a therapeutic compound, which is produced in therapeutically effective amounts or at a therapeutically useful time. It may also encode a product, such as a peptide or RNA, that in some manner mediates, directly or indirectly, expression of a therapeutic product. Expression of the nucleic acid by the target cells within an organism afflicted with a disease or disorder thereby provides for modulation of the disease or disorder. The nucleic acid encoding the therapeutic product may be modified prior to introduction into the cells of the afflicted host in order to enhance or otherwise alter the product or expression thereof.

For use in gene therapy, cells can be transfected *in vitro*, followed by introduction of the transfected cells into an organism. This is often referred to as *ex vivo* gene therapy. Alternatively, the cells can be transfected directly *in vivo* within an organism.

As used herein, therapeutic agents include, but are not limited to, growth factors, antibodies, cytokines, such as tumor necrosis factors and interleukins, and cytotoxic agents and other agents disclosed herein and

known to those of skill in the art. Such agents include, but are not limited to, tumor necrosis factor, α-interferon, β-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator; or, biological response modifiers such as, for example, lymphokines, interleukin- I (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), granulocyte macrophage colony stimulating factor (GMCSF), granulocyte colony stimulating factor (G-CSF), erythropoietin (EPO), pro-coagulants such as tissue factor and tissue factor variants, pro-apoptotic agents such FAS-ligand, fibroblast growth factors (FGF), nerve growth factor and other growth factors.

As used herein, a therapeutically effective product is a product that is encoded by heterologous DNA that, upon introduction of the DNA into a host, a product is expressed that effectively ameliorates or eliminates the symptoms, manifestations of an inherited or acquired disease or that cures the disease.

As used herein, transgenic plants and animals refer to plants and animals in which heterologous or foreign nucleic acid is expressed or in which the expression of a gene naturally present in the plant or animal has been altered by virtue of introduction of heterologous or foreign nucleic acid.

As used herein, IRES (internal ribosome entry site; see, *e.g.*, SEQ ID No. 27 and nucleotides 2736-3308 SEQ ID No. 28) refers to a region of a nucleic acid molecule, such as an mRNA molecule, that allows internal ribosome entry sufficient to initiate translation, which initiation can be detected in an assay for cap-independent translation (see, *e.g.*, U.S. Patent No. 6,171,821). The presence of an IRES within an mRNA molecule allows cap-independent translation of a linked protein-encoding sequence that otherwise would not be translated.

Internal ribosome entry site (IRES) elements were first identified in picornaviruses, which elements are considered the paradigm for cap-independent translation. The 5' UTRs of all picornaviruses are long and mediate translational initiation by directly recruiting and binding ribosomes, thereby circumventing the initial cap-binding step. IRES elements are frequently found in viral mRNA, they are rare in non-viral mRNA. Among non-viral mRNA molecules that contain functional IRES elements in their respective 5' UTRs are those encoding immunoglobulin heavy chain binding protein (BiP) (Macejak *et al.* (1991) *Nature 353*:90-94); Drosophila Antennapedia (Oh *et al.* (1992) *Genes Dev, 6*:1643-1653); D. Ultrabithorax (Ye *et al.* (1997) *Mol. Cell Biol. 17*:1714-21); fibroblast growth factor 2 (Vagner *et al.* (1995) *Mol. Cell Biol. 15*:35-44); initiation factor eIF4G (Gan *et al.* (1998) *J. Biol. Chem. 273*:5006-5012); proto-oncogene c-myc (Nanbru *et al.* (1995) *J. Biol. Chem. 272*:32061-32066; Stoneley (1998) *Oncogene 16*:423-428); $IRES_H$; from the 5'UTR of NRF1 gene (Oumard et al. (2000) *Mol. and Cell Biol.*, 20(8):2755-2759); and vascular endothelial growth factor (VEGF) (Stein *et al.* (1998) *Mol. Cell Biol. 18*:3112-9).

As used herein, a promoter, with respect to a region of DNA, refers to a sequence of DNA that contains a sequence of bases that signals RNA polymerase to associate with the DNA and initiate transcription of RNA (such as pol II for mRNA) from a template strand of the DNA. A promoter thus generally regulates transcription of DNA into mRNA. A particular promoter provided herein is the Ferritin heavy chain promoter (excluding the Iron Response Element, located in the 5'UTR), which was joined to the 37bp Fer-1 enhancer element. This promoter is set forth as SEQ ID NO:128. The endogenous Fer-1 enhancer element is located upstream of the Fer-1 promoter (e.g., a Fer-1 oligo was cloned proximal to the core promoter).

As used herein, isolated, substantially pure nucleic acid, such as, for example, DNA, refers to nucleic acid fragments purified according to standard techniques employed by those skilled in the art, such as that found in Sambrook *et al.* ((2001) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 3rd edition).

As used herein, expression refers to the transcription and/or translation of nucleic acid. For example, expression can be the transcription of a gene that may be transcribed into an RNA molecule, such as a messenger RNA (mRNA) molecule. Expression may further include translation of an RNA molecule and translated into peptides, polypeptides, or proteins. If the nucleic acid is derived from genomic DNA, expression may, if an appropriate eukaryotic host cell or organism is selected, include splicing of the mRNA. With respect to an antisense construct, expression may refer to the transcription of the antisense DNA.

As used herein, vector or plasmid refers to discrete elements that are used to introduce heterologous nucleic acids into cells for either expression of the heterologous nucleic acid or for replication of the heterologous nucleic acid. Selection and use of such vectors and plasmids are well within the level of skill of the art.

As used herein, transformation/transfection refers to the process by which nucleic acid is introduced into cells. The terms transfection and transformation refer to the taking up of exogenous nucleic acid, e.g., an expression vector, by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, by *Agrobacterium*-mediated transformation, protoplast transformation (including polyethylene glycol (PEG)-mediated transformation, electroporation, protoplast fusion, and microcell fusion), lipid-mediated delivery, liposomes, electroporation,

sonoporation, microinjection, particle bombardment and silicon carbide whisker-mediated transformation and combinations thereof (see, *e.g.*, Paszkowski *et al.* (1984) *EMBO J. 3*:2717-2722; Potrykus *et al.* (1985) *Mol. Gen. Genet. 199*:169-177; Reich *et al.* (1986) *Biotechnology 4*:1001-1004; Klein *et al.* (1987) *Nature 327*:70-73; U.S. Patent No. 6,143,949; Paszkowski *et al.* (1989) *in Cell Culture and Somatic Cell Genetics of Plants, Vol. 6*, Molecular Biology of Plant Nuclear Genes, eds. Schell, J and Vasil, L.K. Academic Publishers, San Diego, California, p. 52-68; and Frame *et al.* (1994) *Plant J. 6*:941-948), direct uptake using calcium phosphate (CaPO4; see,*e.g.*, Wigler *et al.* (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*:1373-1376), polyethylene glycol (PEG)-mediated DNA uptake, lipofection (see, *e.g.*, Strauss (1996) *Meth. Mol. Biol. 54*:307-327), microcell fusion (see, EXAMPLES, see, also Lambert (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*:5907-5911; U.S. Patent No. 5,396,767, Sawford *et al.* (1987) *Somatic Cell Mol. Genet. 13*:279-284; Dhar *et al.* (1984) *Somatic Cell Mol. Genet. 10*:547-559; and McNeill-Killary *et al.* (1995) *Meth. Enzymol. 254*:133-152), lipid-mediated carrier systems (see, e.g., Teifel *et al.* (1995) *Biotechniques 19*:79-80; Albrecht *et al.* (1996) *Ann. Hematol. 72*:73-79; Holmen *et al.* (1995) *In Vitro Cell Dev. Biol. Anim. 31*:347-351; Remy *et al.* (1994) *Bioconjug. Chem. 5*:647-654; Le Bolch *et al.* (1995) *Tetrahedron Lett. 36*:6681-6684; Loeffler *et al.* (1993) *Meth. Enzymol. 217*:599-618) or other suitable method. Methods for delivery of *ACes* are described in copending U.S. application Serial No. 09/815,979. Successful transfection is generally recognized by detection of the presence of the heterologous nucleic acid within the transfected cell, such as, for example, any visualization of the heterologous nucleic acid or any indication of the operation of a vector within the host cell.

As used herein, "delivery," which is used interchangeably with "transfection," refers to the process by which exogenous nucleic acid molecules are transferred into a cell such that they are located inside the cell. Delivery of nucleic acids is a distinct process from expression of nucleic acids.

As used herein, injected refers to the microinjection, such as by use of a small syringe, needle, or pipette, for injection of nucleic acid into a cell.

As used herein, substantially homologous DNA refers to DNA that includes a sequence of nucleotides that is sufficiently similar to another such sequence to form stable hybrids, with each other or a reference sequence, under specified conditions.

It is well known to those of skill in this art that nucleic acid fragments with different sequences may, under the same conditions, hybridize detectably to the same "target" nucleic acid. Two nucleic acid fragments hybridize detectably, under stringent conditions over a sufficiently long hybridization period, because one fragment contains a segment of at least about 10, 14 or 16 or more nucleotides in a sequence that is complementary (or nearly complementary) to a substantially contiguous sequence of at least one segment in the other nucleic acid fragment. If the time during which hybridization is allowed to occur is held constant, at a value during which, under preselected stringency conditions, two nucleic acid fragments with complementary base-pairing segments hybridize detectably to each other, departures from exact complementarity can be introduced into the base-pairing segments, and base-pairing will nonetheless occur to an extent sufficient to make hybridization detectable. As the departure from complementarity between the base-pairing segments of two nucleic acids becomes larger, and as

conditions of the hybridization become more stringent, the probability decreases that the two segments will hybridize detectably to each other.

Two single-stranded nucleic acid segments have "substantially the same sequence", if (a) both form a base-paired duplex with the same segment, and (b) the melting temperatures of the two duplexes in a solution of 0.5 X SSPE differ by less than 10°C. If the segments being compared have the same number of bases, then to have "substantially the same sequence", they will typically differ in their sequences at fewer than 1 base in 10. Methods for determining melting temperatures of nucleic acid duplexes are well known (see, *e.g.*, Meinkoth *et al.* (1984) *Anal. Biochem.* *138*:267-284 and references cited therein).

As used herein, a nucleic acid probe is a DNA or RNA fragment that includes a sufficient number of nucleotides to specifically hybridize to DNA or RNA that includes complementary or substantially complementary sequences of nucleotides. A probe may contain any number of nucleotides, from as few as about 10 and as many as hundreds of thousands of nucleotides. The conditions and protocols for such hybridization reactions are well known to those of skill in the art as are the effects of probe size, temperature, degree of mismatch, salt concentration and other parameters on the hybridization reaction. For example, the lower the temperature and higher the salt concentration at which the hybridization reaction is carried out, the greater the degree of mismatch that may be present in the hybrid molecules.

To be used as a hybridization probe, the nucleic acid is generally rendered detectable by labeling it with a detectable moiety or label, such as ^{32}P, ^{3}H and ^{14}C, or by other means, including chemical labeling, such as by nick-translation in the presence of deoxyuridylate biotinylated at the 5′-position of the uracil moiety. The resulting probe includes the biotinylated uridylate in place of thymidylate residues and can be detected

(via the biotin moieties) by any of a number of commercially available detection systems based on binding of streptavidin to the biotin. Such commercially available detection systems can be obtained, for example, from Enzo Biochemicals, Inc. (New York, NY). Any other label known to those of skill in the art, including non-radioactive labels, may be used as long as it renders the probes sufficiently detectable, which is a function of the sensitivity of the assay, the time available (for culturing cells, extracting DNA, and hybridization assays), the quantity of DNA or RNA available as a source of the probe, the particular label and the means used to detect the label.

Once sequences with a sufficiently high degree of homology to the probe are identified, they can readily be isolated by standard techniques (see, *e.g.*, Sambrook *et al.* (2001) *Molecular Cloning: A Laboratory Manual*, 3rd Edition, Cold Spring Harbor Laboratory Press).

As used herein, conditions under which DNA molecules form stable hybrids are considered substantially homologous, and a DNA or nucleic acid homolog refers to a nucleic acid that includes a preselected conserved nucleotide sequence, such as a sequence encoding a polypeptide. By the term "substantially homologous" is meant having at least 75%, preferably 80%, preferably at least 90%, most preferably at least 95% homology therewith or a less percentage of homology or identity and conserved biological activity or function.

The terms "homology" and "identity" are often used interchangeably. In this regard, percent homology or identity may be determined, for example, by comparing sequence information using a GAP computer program. The GAP program utilizes the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443 (1970), as revised by Smith and Waterman (*Adv. Appl. Math.* 2:482 (1981). Briefly, the GAP program defines similarity as the number of aligned symbols (i.e.,

nucleotides or amino acids) which are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program may include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.* 14:6745 (1986), as described by Schwartz and Dayhoff, eds., *ATLAS OF PROTEIN SEQUENCE AND STRUCTURE*, National Biomedical Research Foundation, pp. 353-358 (1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

By sequence identity, the number of conserved amino acids are determined by standard alignment algorithms programs, and are used with default gap penalties established by each supplier. Substantially homologous nucleic acid molecules would hybridize typically at moderate stringency or at high stringency all along the length of the nucleic acid of interest. Preferably the two molecules will hybridize under conditions of high stringency. Also contemplated are nucleic acid molecules that contain degenerate codons in place of codons in the hybridizing nucleic acid molecule.

Whether any two nucleic acid molecules have nucleotide sequences that are at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% "identical" can be determined using known computer algorithms such as the "FAST A" program, using for example, the default parameters as in Pearson and Lipman, *Proc. Natl. Acad. Sci. USA 85*:2444 (1988). Alternatively the BLAST function of the National Center for Biotechnology Information database may be used to determine relative sequence identity.

In general, sequences are aligned so that the highest order match is obtained. "Identity" *per se* has an art-recognized meaning and can be

calculated using published techniques. (See, *e.g.*: *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). While there exist a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to skilled artisans (Carillo, H. & Lipton, D., *SIAM J Applied Math 48*:1073 (1988)). Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in Guide to Huge Computers, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo, H. & Lipton, D., *SIAM J Applied Math 48*:1073 (1988). Methods to determine identity and similarity are codified in computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCG program package (Devereux, J., *et al., Nucleic Acids Research 12(l)*:387 (1984)), BLASTP, BLASTN, FASTA (Atschul, S.F., *et al., J Molec Biol 215*:403 (1990)).

Therefore, as used herein, the term "identity" represents a comparison between a test and a reference polypeptide or polynucleotide. For example, a test polypeptide may be defined as any polypeptide that is 90% or more identical to a reference polypeptide.

As used herein, the term at least "90% identical to" refers to percent identities from 90 to 99.99 relative to the reference polypeptides. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polynucleotide length of

100 amino acids are compared. No more than 10% (i.e., 10 out of 100) amino acids in the test polypeptide differs from that of the reference polypeptides. Similar comparisons may be made between a test and reference polynucleotides. Such differences may be represented as point mutations randomly distributed over the entire length of an amino acid sequence or they may be clustered in one or more locations of varying length up to the maximum allowable, e.g. 10/100 amino acid difference (approximately 90% identity). Differences are defined as nucleic acid or amino acid substitutions, or deletions.

As used herein: stringency of hybridization in determining percentage mismatch encompass the following conditions or equivalent conditions thereto:

1) high stringency: 0.1 x SSPE or SSC, 0.1% SDS, 65°C
2) medium stringency: 0.2 x SSPE or SSC, 0.1% SDS, 50°C
3) low stringency: 1.0 x SSPE or SSC, 0.1% SDS, 50°C

or any combination of salt and temperature and other reagents that result in selection of the same degree of mismatch or matching. Equivalent conditions refer to conditions that select for substantially the same percentage of mismatch in the resulting hybrids. Additions of ingredients, such as formamide, Ficoll, and Denhardt's solution affect parameters such as the temperature under which the hybridization should be conducted and the rate of the reaction. Thus, hybridization in 5 X SSC, in 20% formamide at 42° C is substantially the same as the conditions recited above hybridization under conditions of low stringency. The recipes for SSPE, SSC and Denhardt's and the preparation of deionized formamide are described, for example, in Sambrook *et al.* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Chapter 8; see, Sambrook *et al.*, vol. 3, p. B.13, see, also, numerous catalogs that describe commonly used laboratory solutions. It is understood that

equivalent stringencies may be achieved using alternative buffers, salts and temperatures. As used herein, all assays and procedures, such as hybridization reactions and antibody-antigen reactions, unless otherwise specified, are conducted under conditions recognized by those of skill in the art as standard conditions.

As used herein, conservative amino acid substitutions, such as those set forth in Table 1, are those that do not eliminate biological activity. Suitable conservative substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, *e.g.*, Watson *et al. Molecular Biology of the Gene*, 4th Edition, 1987, The Bejacmin/Cummings Pub. co., p.224). Conservative amino acid substitutions are made, for example, in accordance with those set forth in TABLE 1 as follows:

TABLE 1

Original residue	Conservative substitution
Ala (A)	Gly; Ser, Abu
Arg (R)	Lys, orn
Asn (N)	Gln; His
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
Gly (G)	Ala; Pro
His (H)	Asn; Gln
Ile (I)	Leu; Val; Met; Nle; Nva
Leu (L)	Ile; Val; Met; Nle; Nva
Lys (K)	Arg; Gln; Glu
Met (M)	Leu; Tyr; Ile; NLe Val
Ornithine	Lys; Arg
Phe (F)	Met; Leu; Tyr
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp; Phe
Val (V)	Ile; Leu; Met; Nle; Nva

Other substitutions are also permissible and may be determined empirically or in accord with known conservative substitutions.

As used herein, the amino acids, which occur in the various amino acid sequences appearing herein, are identified according to their well-known, three-letter or one-letter abbreviations. The nucleotides, which occur in the various DNA fragments, are designated with the standard single-letter designations used routinely in the art.

As used herein, a splice variant refers to a variant produced by differential processing of a primary transcript of genomic DNA that results in more than one type of mRNA.

As used herein, a probe or primer based on a nucleotide sequence includes at least 10, 14, 16, 30 or 100 contiguous nucleotides from the reference nucleic acid molecule.

As used herein, recombinant production by using recombinant DNA methods refers to the use of the well known methods of molecular biology for expressing proteins encoded by cloned DNA.

As used herein, biological activity refers to the *in vivo* activities of a compound or physiological responses that result upon *in vivo* administration of a compound, composition or other mixture. Biological activity, thus, encompasses therapeutic effects and pharmaceutical activity of such compounds, compositions and mixtures. Biological activities may be observed in *in vitro* systems designed to test or use such activities. Thus, for purposes herein the biological activity of a luciferase is its oxygenase activity whereby, upon oxidation of a substrate, light is produced.

The terms substantially identical or similar varies with the context as understood by those skilled in the relevant art and generally means at least 40, 60, 80, 90, 95 or 98%.

As used herein, substantially identical to a product means sufficiently similar so that the property is sufficiently unchanged so that the substantially identical product can be used in place of the product.

As used herein, substantially pure means sufficiently homogeneous to appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound may, however, be a mixture of stereoisomers or isomers. In such instances, further purification might increase the specific activity of the compound.

As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. The vectors typically remain episomal, but may be designed to effect integration of a gene or portion thereof into a chromosome of the genome. Also contemplated are vectors that are artificial chromosomes, such as yeast artificial chromosomes and mammalian artificial chromosomes. Selection and use of such vehicles are well known to those of skill in the art. An expression vector includes vectors capable of expressing DNA that is operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector

refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

As used herein, protein-binding-sequence refers to a protein or peptide sequence that is capable of specific binding to other protein or peptide sequences generally, to a set of protein or peptide sequences or to a particular protein or peptide sequence.

As used herein, a composition refers to any mixture of two or more ingredients. It may be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous or any combination thereof.

As used herein, a combination refers to any association between two or more items.

As used herein, fluid refers to any composition that can flow. Fluids thus encompass compositions that are in the form of semi-solids, pastes, solutions, aqueous mixtures, gels, lotions, creams and other such compositions.

As used herein, a cellular extract refers to a preparation or fraction that is made from a lysed or disrupted cell.

As used herein, the term "subject" refers to animals, plants, insects, and birds and other phyla, genera and species into which nucleic acid molecules may be introduced. Included are higher organisms, such as mammals, fish, insects and birds, including humans, primates, cattle, pigs, rabbits, goats, sheep, mice, rats, guinea pigs, hamsters, cats, dogs, horses, chicken and others.

As used herein, flow cytometry refers to processes that use a laser based instrument capable of analyzing and sorting out cells and or chromosomes based on size and fluorescence.

As used herein, the abbreviations for any protective groups, amino acids and other compounds, are, unless indicated otherwise, in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature (see, (1972) *Biochem.* *11*:942-944).

B. Recombination systems

Site-specific recombination systems typically contain three elements: a pair of DNA sequences (the site-specific recombination sequences) and a specific enzyme (the site-specific recombinase). The site-specific recombinase catalyzes a recombination reaction between two site-specific recombination sequences.

A number of different site-specific recombinase systems are available and/or known to those of skill in the art, including, but not limited to: the Cre/*lox* recombination system using CRE recombinase (see, *e.g.*, SEQ ID Nos. 58 and 59) from the Escherichia coli phage P1 (see, *e.g.*, Sauer (1993) *Methods in Enzymology 225*:890-900; Sauer *et al.* (1990) *The New Biologist 2*:441-449), Sauer (1994) *Current Opinion in Biotechnology 5*:521-527; Odell *et al.* (1990) *Mol Gen Genet. 223*:369-378; Lasko *et al.* (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*:6232-6236; U.S. Patent No. 5,658,772), the FLP/FRT system of yeast using the FLP recombinase (see, SEQ ID Nos. 60 and 61) from the 2μ episome of Saccharomyces cerevisiae (Cox (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*:4223; Falco *et al.* (1982) *Cell 29*:573-584; Golic *et al.* (1989) *Cell*59:499-509; U.S. Patent No. 5,744,336), the resolvases, including Gin recombinase of phage Mu (Maeser *et al.* (1991) *Mol Gen Genet. 230*:170-176; Klippel, A. et al (1993) *EMBO J. 12*:1047-1057; see, *e.g.*,

SEQ ID Nos. 64-67), Cin, Hin, *αδ* Tn3; the Pin recombinase of *E. coli* (see, *e.g.*, SEQ ID Nos. 68 and 69; Enomoto *et al.* (1983) *J Bacteriol. 6*:663-668), the R/RS system of the pSR1 plasmid of Zygosaccharomyces rouxii (Araki *et al.* (1992) *J. Mol. Biol.* 225:25-37; Matsuzaki *et al.* (1990) *J. Bacteriol. 172*: 610-618) and site-specific recombinases from Kluyveromyces drosophilarium (Chen *et al.* (1986) *Nucleic Acids Res. 314*:4471-4481) and Kluyveromyces waltii (Chen *et al.* (1992) *J. Gen. Microbiol. 138*:337-345). Other systems are known to those of skill in the art (Stark *et al. Trends Genet. 8*:432-439; Utatsu *et al.* (1987) *J. Bacteriol. 169*:5537-5545; see, also, U.S. Patent No. 6,171,861).

Members of the highly related family of site-specific recombinases, the resolvase family, such as *γδ*, Tn3 resolvase, Hin, Gin, and Cin are also available. Members of this family of recombinases are typically constrained to intramolecular reactions (e.g., inversions and excisions) and can require host-encoded factors. Mutants have been isolated that relieve some of the requirements for host factors (Maeser *et al.* (1991) *Mol. Gen. Genet. 230*:170-176), as well as some of the constraints of intramolecular recombination (see, U.S. Patent No. 6,171,861).

The bacteriophage P1 Cre/lox and the yeast FLP/FRT systems are particularly useful systems for site-specific integration, inversion or excision of heterologous nucleic acid into, and out of, chromosomes, particularly *ACes* as provided herein. In these systems a recombinase (Cre or FLP) interacts specifically with its respective site-specific recombination sequence (lox or FRT, respectively) to invert or excise the intervening sequences. The sequence for each of these two systems is relatively short (34 bp for lox and 47 bp for FRT).

The FLP/FRT recombinase system has been demonstrated to function efficiently in plant cells (U.S. Patent No. 5,744,386), and, thus, can be used for producing plant artificial chromosome platforms. In

general, short incomplete FRT sites leads to higher accumulation of excision products than the complete full-length FRT sites. The system catalyzes intra- and intermolecular reactions, and, thus, can be used for DNA excision and integration reactions. The recombination reaction is reversible and this reversibility can compromise the efficiency of the reaction in each direction. Altering the structure of the site-specific recombination sequences is one approach to remedying this situation. The site-specific recombination sequence can be mutated in a manner that the product of the recombination reaction is no longer recognized as a substrate for the reverse reaction, thereby stabilizing the integration or excision event.

In the Cre-lox system, discovered in bacteriophage P1, recombination between loxP sites occurs in the presence of the Cre recombinase (see, *e.g.*, U.S. Patent No. 5,658,772). This system can be used to insert, invert or excise nucleic acid located between two lox sites. Cre can be expressed from a vector. Since the lox site is an asymmetrical nucleotide sequence, lox sites on the same DNA molecule can have the same or opposite orientation with respect to each other. Recombination between lox sites in the same orientation results in a deletion of the DNA segment located between the two lox sites and a connection between the resulting ends of the original DNA molecule. The deleted DNA segment forms a circular molecule of DNA. The original DNA molecule and the resulting circular molecule each contain a single lox site. Recombination between lox sites in opposite orientations on the same DNA molecule result in an inversion of the nucleotide sequence of the DNA segment located between the two lox sites. In addition, reciprocal exchange of DNA segments proximate to lox sites located on two different DNA molecules can occur. All of these recombination events are catalyzed by the product of the Cre coding region.

Any site-specific recombinase system known to those of skill in the art is contemplated for use herein. It is contemplated that one or a plurality of sites that direct the recombination by the recombinase are introduced into an artificial chromosome to produce platform *ACes*. The resulting platform *ACes* are introduced into cells with nucleic acid encoding the cognate recombinase, typically on a vector, and nucleic acid encoding heterologous nucleic acid of interest linked to the appropriate recombination site for insertion into the platform *ACes*. The recombinase-encoding-nucleic acid may be introduced into the cells on the same vector, or a different vector, encoding the heterologous nucleic acid.

An *E. coli* phage lambda integrase system for *ACes* platform engineering and for artificial chromosome engineering is provided (Lorbach *et al.* (2000) *J. Mol. Biol 296*:1175-1181). The phage lambda integrase (Landy, A. (1989) *Annu. Rev. Biochem. 58*:913-94) is adapted herein and the cognate *att* sites are provided. Chromosomes, including *ACes*, engineered to contain one or a plurality of *att* sites are provided, as are vectors encoding a mutant integrase that functions in the absence other factors. Methods using the modified chromosomes and vectors for introduction of heterologous nucleic acid are also provided.

For purposes herein, one or more of the sites (e.g., a single site or a pair of sites) required for recombination are introduced into an artificial chromosome, such as an *ACes* chromosome. The enzyme for catalyzing site-directed recombination is introduced with the DNA of interest, or separately, or is engineered onto the artificial chromosome under the control of a regulatable promoter.

As described herein, artificial chromosome platforms containing one or multiple recombination sites are provided. The methods and resulting products are exemplified with the lambda phage Att/Int system, but

similar methods may be used for production of *ACes* platforms with other recombination systems.

The Att/Int system and vectors provided herein are not only intended for engineering *ACes* platforms, but may be used to engineer an Att/Int system into any chromosome. Introduction of att sites into a chromosome will permit engineering of natural chromosomes, such as by permitting targeted integration genes or regulatory regions, and by controlled excision of selected regions. For example, genes encoding a particular trait may be added to a chromosome, such as plant chromosome engineered to contain one or plurality of *att* sites. Such chromosomes may be used for screening DNA to identify genes. Large pieces of DNA can be introduced into cells and the cells screened phenotypically to select those having the desired trait.

C. Platforms

Provided herein are platform artificial chromosomes (platform *ACes*) containing single or multiple site-specific recombination sites. Chromosome-based platform technology permits efficient and tractable engineering and subsequent expression of multiple gene targets. Methods are provided that use DNA vectors and fragments to create platform artificial chromosomes, including animal, particularly mammalian, artificial chromosomes, and plant artificial chromosomes. The artificial chromosomes contain either single or multiple sequence-specific recombination sites suitable for the placement of target gene expression vectors onto the platform chromosome. The engineered chromosome-based platform *ACes* technology is applicable for methods, including cellular and transgenic protein production, transgenic plant and animal production and gene therapy. The platform *ACes* are also useful for producing a library of *ACes* comprising random portions of a given genome (e.g., a mammalian, plant or prokaryotic genome) for genomic

screening; as well as a library of cells comprising different and/or mutually exclusive *ACes* therein.

Exemplary of artificial chromosome platforms are those based on *ACes*. *ACes* artificial chromosomes are non-viral, self-replicating nucleic acid molecules that function as a natural chromosome, having all the elements required for normal chromosomal replication and maintenance within the cell nucleus. *ACes* artificial chromosomes do not rely on integration into the genome of the cell to be effective, and they are not limited by DNA carrying capacity and as such the therapeutic gene(s) of interest, including regulatory sequences, can be engineered into the *ACes*. In addition, *ACes* are stable *in vitro* and *in vivo* and can provide predictable long-term gene expression. Once engineered and delivered to the appropriate cell or embryo, *ACes* work independently alongside host chromosomes, for *ACes* that are predominantly heterochromatin producing only the products (proteins) from the genes it carries. As provided herein *ACes* are modified by introduction of recombination site(s) to provide a platform for ready introduction of heterologous nucleic acid. The *ACes* platforms can be used for production of transgenic animals and plants; as vectors for genetic therapy; for use as protein production systems; for animal models to identify and target new therapeutics; in cell culture for the development and production of therapeutic proteins; and for a variety of other applications.

1. Generation of artificial chromosomes

Artificial chromosomes may be generated by any method known to those of skill in the art. Of particular interest herein are the *ACes* artificial chromosomes, which contain a repeated unit. Methods for production of *ACes* are described in detail in U.S. Patent Nos. 6,025,155 and 6,077,697, which, as with all patents, applications, publications and other disclosure, are incorporated herein in their entirety.

Generation of *de novo* *ACes*.

ACes can be generated by cotransfecting exogenous DNA—such as a mammary tissue specific DNA cassette including the gene sequences for a therapeutic protein, with a rDNA fragment and a drug resistance marker gene into the desired eukaryotic cell, such as plant or animal cells, such as murine cells *in vitro*. DNA with a selectable or detectable marker is introduced, and can be allowed to integrate randomly into pericentric heterochromatin or can be targeted to pericentric heterochromatin, such as that in rDNA gene arrays that reside on acrocentric chromosomes, such as the short arms of acrocentric chromosomes. This integration event activates the "megareplicator" sequence and amplifies the pericentric heterochromatin and the exogenous DNA, and duplicates a centromere. Ensuing breakage of this "dicentric" chromosome can result in the production of daughter cells that contain the substantially-original chromosome and the new artificial chromosome. The resulting *ACes* contain all the essential elements needed for stability and replication in dividing cells—centromere, origins of replications, and telomeres. *ACes* have been produced that express marker genes (lacZ, green fluorescent protein, neomycin-resistance, puromycin-resistance, hygromycin-resistance) and genes of interest. Isolated *ACes*, for example, have been successfully transferred intact to rodent, human, and bovine cells by electroporation, sonoporation, microinjection, and transfection with lipids and dendrimers.

To render the creation of *ACes* with desired genes more tractable and efficient, "platform" *ACes* (platform-*ACes*) can be produced that contain defined DNA sequences for enzyme-mediated homologous DNA recombination, such as by Cre or FLP recombinases (Bouhassira *et al.* (1996) *Blood 88(supplement 1)*:190a; Bouhassira *et al.* (1997) *Blood, 90*:3332-3344; Siebler *et al.* (1997) *Biochemistry: 36*:1740-1747;

Siebler *et al.* (1998) *Biochemistry 37*: 6229-6234; and Bethke *et al.* (1997) *Nucl. Acids Res. 25*:2828-2834), and as exemplified herein the lambda phage integrase. A *lox* site contains two 13 bp inverted repeats to which Cre-recombinase binds and an intervening 8 bp core region. Only pairs of sites having identity in the central 6 bp of the core region are proficient for recombination; sites having non-identical core sequences (heterospecific *lox* sites) do not efficiently recombine with each other (Hoess *et al.* (1986) *Nucleic Acids Res. 14*:2287-2300).

Generating acrocentric chromosomes for plant artificial chromosome formation.

In human and mouse cells *de novo* formation of a satellite DNA based artificial chromosome (SATAC, also referred to as *ACes*) can occur in an acrocentric chromosome where the short arm contains only pericentric heterochromatin, the rDNA array, and telomere sequences. Plant species may not have any acrocentric chromosomes with the same physical structure described, but "megareplicator" DNA sequences reside in the plant rDNA arrays, also known as the nucleolar organizing regions (NOR). A structure like those seen in acrocentric mammalian chromosomes can be generated using site-specific recombination between appropriate arms of plant chromosomes.

Approach

Qin *et al.* ((1994) *Proc. Natl. Acad. Sci. U.S.A. 91*:1706-1710, 1994) describes crossing two *Nicotiana tabacum* transgenic plants. One plant contains a construct encoding a promoterless hygromycin-resistance gene preceded by a *lox* site (*lox-hpt*), the other plant carries a construct containing a cauliflower mosaic virus 35S promoter linked to a *lox* sequence and the *cre* DNA recombinase coding region (35S-*lox-cre*). The constructs were introduced separately by infecting leaf explants with *agrobacterium tumefaciens* which carries the kanamycin-resistance gene

(Kan^R). The resultant Kan^R transgenic plants were crossed. Plants that carried the appropriate DNA recombination event were identified by hygromycin-resistance.

Modification of the above for generation of *ACes*

The Kan^R cultivars are initially screened, such as by FISH, to identify two sets of candidate transgenic plants. One set has one construct integrated in regions adjacent to the pericentric heterochromatin on the short arm of any chromosome. The second set of candidate plants has the other construct integrated in the NOR region of appropriate chromosomes. To obtain reciprocal translocation both sites must be in the same orientation. Therefore a series of crosses are required, Kan^R plants generated, and FISH analyses performed to identify the appropriate "acrocentric" plant chromosome for *de novo* plant *ACes* formation.

2. Bacteriophage lambda integrase-based site-specific recombination system

An integral part of the platform technology includes a site-specific recombination system that allows the placement of selected gene targets or genomic fragments onto the platform chromosomes. Any such system may be used. In particular, a method is provided for insertion of additional DNA fragments into the platform chromosome residing in the cell via sequence-specific recombination using the recombinase activity of the bacteriophage lambda integrase. The lambda integrase system is exemplary of the recombination systems contemplated for *ACes*. Any known recombination system, including any described herein, particularly any that operates without the need for additional factors or that, by virtue of mutation, does not require additional factors, is contemplated.

As noted the lambda integrase system provided herein can be used with natural chromosomes and artificial chromosomes in addition to *ACes*. Single or a plurality of recombination sites, which may be the same or different, are introduced into artificial chromosomes to produce artificial chromosome platforms.

3. Creation of bacteriophage lambda integrase site-specific recombination system

The lambda phage-encoded integrase (designated Int) is a prototypical member of the integrase family. Int effects integration and excision of the phage in and out of the *E. coli* genome via recombination between pairs of attachment sites designated *att*B/*att*P and *att*L/*att*R. Each *att* site contains two inverted 9 base pair core Int binding sites and a 7 base pair overlap region that is identical in wild-type *att* sites. Each site, except for *att*B contains additional Int binding sites. In flanking regions, there are recognition sequences for accessory DNA binding proteins, such as integration host factor (IHF), factor for inversion stimulation (FIS) and the phage encoded excision protein (XIS). Except for *att*B, Int is a heterobivalent DNA-binding protein and, with assistance from the accessory proteins and negative DNA supercoiling, binds simultaneously to core and arm sites within the same *att* site.

Int, like Cre and FLP, executes an ordered sequential pair of strand exchanges during integrative and excisive recombination. The natural pairs of target sequences for Int, *att*B and *att*P or *att*L and *att*R are located on the same or different DNA molecules resulting in intra or intermolecular recombination, respectively. For example, intramolecular recombination occurs between inversely oriented *att*B and *att*P, or between *att*L and *att*R sequences, respectively, leading to inversion of the intervening DNA segment.

Like the recombinase systems, such as Cre and FLP, Int directs site-specific recombination. Unlike the other systems, such Cre and FLP, Int generally requires additional protein factors for integrative and excisive recombination and negative supercoiling for integrative recombination. Hence, the Int system had not been used in eukaryotic targeting systems.

Mutant Int proteins, designated Int-h (E174K) and a derivative thereof Int-h/218(E174K/E218K) do not require accessory proteins to perform intramolecular integrative and excisive recombination in co-transfection assays in human cells (Lorbach *et al.* (2000) *J Mol. Biol. 296*:1175-1181); wild-type Int does not catalyze intramolecular recombination in human cells harboring target sites *att*B and *att*P. Hence it had been demonstrated that mutant Int can catalyze factor-independent recombination events in human cells.

There has been no demonstration by others that this system can be used for engineering of eukaryotic genomes or chromosomes. Provided herein are chromosomes, including artificial chromosomes, such as but not limited to *ACes* that contain *att* sites (e.g., platform *ACes*), and the use of such chromosomes for targeted integration of heterologous DNA into such chromosomes in eukaryotic cells, including animal, such as rodent and human, and plant cells. Mutant Int provided herein is shown to effect site-directed recombination between sites in artificial chromosomes and vectors containing cognate sites.

An additional component of the chromosome-based platform technology is the site-specific integration of target DNA sequences onto the platform. For this the native bacteriophage lambda integrase has been modified to carry out this sequence specific DNA recombination event in eukaryotic cells. The bacteriophage lambda integrase and its cognate DNA substrate att is a member of the site-specific recombinase family that also includes the bacteriophage P1 Cre/lox system as well as

the *Saccharomyces cerevisiae* 2 micron based FLP/FRT system (see, *e.g.*, Landy (1989) *Ann. Rev. Biochem 58*:913-949; Hoess *et al.* (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*:3398-3402; Broach *et al.* (1982) *Cell 29*:227-234).

By combining DNA endonuclease and DNA ligase activity these recombinases recognize and catalyze DNA exchanges between sequences flanking the recognition site. During the integration of lambda genome into the *E. coli* (lambda recombination) genome, the phage integrase (INT) in association with accessory proteins catalyzes the DNA exchange between the attP site of the phage genome and the attB site of the bacterial genome resulting in the formation of attL and attR sites (Figure 6). The engineered bacteriophage lambda integrase has been produced herein to carry out an intermolecular DNA recombination event between an incoming DNA molecule (primarily on a vector containing the bacterial attB site) and the chromosome-based platform carrying the lambda attP sequence independent of lambda bacteriophage or bacterial accessory proteins.

In contrast to the bi-directional Cre/lox and FLP/FRT system, the engineered lambda recombination system derived for chromosome-based platform technology is advantageously unidirectional because accessory proteins, which are absent, are required for excision of integrated nucleic acid upon further exposure to the lambda Int recombinase.

4. Creation of platform chromosome containing single or multiple sequence-specific recombination sites

a. Multiple sites

For the creation of a platform chromosome containing multiple, sequence-specific recombination sites, artificial chromosomes are produced as depicted in Figure 5 and Example 3. As discussed above, artificial chromosomes can be produced using any suitable methodology,

including those described in U.S. Patent Nos. 5,288,625; 5,712,134; 5,891,691; 6,025,155. Briefly, to prepare artificial chromosomes containing multiple recombination (e.g., integration) sites, nucleic acid (either in the form a one or more plasmids, such as the plasmid pSV40193attPsensePUR set forth in Example 3) is targeted into an amplifiable region of a chromosome, such as the pericentric region of a chromosome. Among such regions are the rDNA gene loci in acrocentric mammalian chromosomes. Hence, targeting nucleic acid for integration into the rDNA region of mammalian acrocentric chromosomes can include the mouse rDNA fragments (for targeting into rodent cell lines) or large human rDNA regions on BAC/PAC vectors (or subclones thereof in standard vectors) for targeting into human acrocentric chromosomes, such as for human gene therapy applications. The targeting nucleic acid generally includes a detectable or selectable marker, such as antibiotic resistance, such as puromycin and hygromycin, a recombination site (such as attP, attB, attL, attR or the like), and/or human selectable markers as required for gene therapy applications. Cells are grown under conditions that result in amplification and ultimately production of *ACes* artificial chromosomes having multiple recombination (e.g.,integration) sites therein. *ACes* having the desired size are selected for further engineering.

b. Creation of platform chromosome containing a single sequence-specific recombination site

In this method a mammalian platform artificial chromosome is generated containing a single sequence-specific recombination site. In the Example below, this approach is demonstrated using a puromycin resistance marker for selection and a mouse rDNA fragment for targeting into the rDNA locus on mouse acrocentric chromosomes. Other selection markers and targeting DNA sequences as desired and known to those of

skill in the art can be used. Additional resistance markers include genes conferring resistance to the antibiotics neomycin, blasticidin, hygromycin and zeocin. For applications, such as gene therapy in which potentially immunogenic responses are to be avoided, host, such as human, derived selectable markers or markers detectable with monoclonal antibodies (MAb) followed by fluorescent activated cell sorting (FACS) can be used. Examples in this class include, but are not limited to: human nerve growth factor receptor (detection with MAb); truncated human growth factor receptor (detection with MAb); mutant human dihydrofolate reductase (DHFR; detectable using a fluorescent methotrexate substrate); secreted alkaline phosphatase (SEAP; detectable with fluorescent substrate); thymidylate synthase (TS; confers resistance to fluorodeoxyuridine); human CAD gene (confers resistance to N-phosphonacetyl-L-aspartate (PALA)).

To construct a platform artificial chromosome with a single site, an *ACes* artificial chromosome (or other artificial chromosome of interest) can be produced containing a selectable marker. A single sequence specific recombination site is targeted onto *ACes* via homologous recombination. For this, DNA sequences containing the site-specific recombination sequence are flanked with DNA sequences homologous to a selected sequence in the chromosome. For example, when using a chromosome containing rDNA or satellite DNA, such DNA can be used as homologous sequences to target the site-specific recombination sequence onto the chromosome. A vector is designed to have these homologous sequences flanking the site-specific recombination site and, after the appropriate restriction enzyme digest to generate free ends of homology to the chromosome, the DNA is transfected into cells harboring the chromosome. After transfection and integration of the site-specific cassette, homologous recombination events onto the platform

chromosome are subcloned and identified, for example by screening single cell subclones via expression of resistance or a fluorescent marker and PCR analysis. In one embodiment, a platform artificial chromosome, such as a platform *ACes*, that contains a single copy of the recombination site is selected. Examples 2B and 2D exemplify the process, and Figure 3 provides a diagram depicting one method for the creation of a platform mammalian chromosome containing a single sequence-specific recombination site.

5. Lambda integrase mediated recombination of target gene expression vector onto platform chromosome

The third component of the chromosome-based platform technology involves the use of target gene expression vectors carrying, for example, genes for gene therapy, genes for transgenic animal or plant production, and those required for cellular protein production of interest. Using lambda integrase mediated site-specific recombination, or any other recombinase-mediated site-specific recombination, the target gene expression vectors are introduced onto the selected chromosome platform. The use of target gene expression vector permits use of the *de novo* generated chromosome-based platforms for a wide range of gene targets. Furthermore, chromosome platforms containing multiple *att*P sites provides the opportunity to incorporate multiple gene targets onto a single platform, thereby providing for expression of multiple gene targets, including the expression of cellular and genetic regulatory genes and the expression of all or parts of metabolic pathways. In addition to expressing small target genes, such as cDNA and hybrid cDNA/artificial intron constructs, the chromosome-based platform can be used for engineering and expressing large genomic fragments carrying target genes along with its endogenous genomic promoter sequences. This is of importance, for example, where the therapy requires precise cell specific

expression and in instances where expression is best achieved from genomic clones rather than cDNA clones. Figure 9 provides a diagram summarizing one embodiment of the chromosome-based technology.

A feature of the target gene expression vector that is of interest to include is a promoterless marker gene, which as exemplified (see, Figure 9) contains an upstream *att*B site (marker 2 on Figure 9). The nucleic acid encoding the marker is not expressed unless it is placed downstream from a promoter sequence. Using the recombinase technology provided herein, such as the lambda integrase technology (λINT_{E174R} on figure 8) provided herein, site-specific recombination between the attB site on the vector and the promoter-attP site (in the "sense" orientation) on the chromosome-based platform results in the expression of marker 2 on the target gene expression vector, thereby providing a positive selection for the lambda INT mediated site-specific recombination event. Site-specific recombination events on the chromosome-based platform versus random integrations next to a promoter in the genome (false positive) can be quickly screened by designing primers to detect the correct event by PCR. Examples of suitable marker 2 genes, include, but are not limited to, genes that confer resistance to toxic compounds or antibiotics, fluorescence activated cell sorting (FACS) sortable cell surface markers and various fluorescent markers. Examples of these genes include, but are not limited to, human L26a^{R} (human homolog of *Saccharomyces cerevisiae* CYH^{R} gene), neomycin, puromycin, blasticidin, CD24 (see, e.g., US Patents 5,804,177 and 6,074,836), truncated CD4, truncated low affinity nerve growth factor receptor (LNGFR), truncated LDL receptor, truncated human growth hormone receptor, GFP, RFP, BFP.

The target gene expression vectors contain a gene (target gene) for expression from the chromosome platform. The target gene can be expressed using various constitutive or regulated promoter systems

across various mammalian species. For the expression of multiple target genes within the same target gene expression vector, the expression of the multiple targets can be coordinately regulated via viral-based or human internal ribosome entry site (IRES) elements (see, *e.g.*, Jackson *et al.* (1990) *Trends Biochem Sci. 15*: 477-83; Oumard *et al.* (2000) *Mol. Cell. Biol. 20*: 2755-2759). Furthermore, using IRES type elements linked to a downstream fluorescent marker, *e.g.*, green, red or blue fluorescent proteins (GFP, RFP, BFP) allows for the identification of high expressing clones from the integrated target gene expression vector.

In certain embodiments described herein, the promoterless marker can be transcriptionally downstream of the heterologous nucleic acid, wherein the heterologous nucleic acid encodes a heterologous protein, and wherein the expression level of the selectable marker is transcriptionally linked to the expression level of the heterologous protein. In addition, the selectable marker and the heterologous nucleic acid can be transcriptionally linked by the presence of a IRES between them. As set forth herein the selectable marker is selected from the group consisting of an antibiotic resistance gene, and a detectable protein, wherein the detectable protein is chromogenic or fluorescent. Expression from the target gene expression vector integrated onto the chromosome-based platform can be further enhanced using genomic insulator/boundary elements. The incorporation of insulator sequences into the target gene expression vector helps define boundaries in chromatin structure and thus minimizes influence of chromatin position effects/gene silencing on the expression of the target gene (Bell *et al.* (1999) *Current Opinion in Genetics and Development 9*:191-198; Emery *et al.* (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*:9150-9155). Examples of insulator elements that can be included onto target gene expression vector in order to optimize expression include, but are not limited to:

1) chicken β-globin HS4 element (Prioleau *et al.* (1999) *EMBO J 18*: 4035-4048);

2) matrix attachment regions (MAR; see, *e.g.*, Ramakrishnan *et al.* (2000) *Mol Cell. Biol. 20*:868-877);

3) scaffold attachment regions (SAR; see, *e.g.*, Auten *et al.* (1999) *Human Gene Therapy 10*:1389-1399); and

4) universal chromatin opening elements (UCOE; WO/0005393 and WO/0224930)

The copy number of the target gene can be controlled by sequentially adding multiple target gene expression vectors containing the target gene onto multiple integration sites on the chromosome platform. Likewise, the copy number of the target gene can be controlled within an individual target gene expression vector by the addition of DNA sequences that promote gene amplification. For example, gene amplification can be induced utilizing the dihydrofolate reductase (DHFR) minigene with subsequent selection with methotrexate (see, *e.g.*, Schimke (1984) *Cell 37*:705-713) or amplification promoting sequences from the rDNA locus (see, *e.g.*, Wegner *et al.* (1989) *Nucl. Acids Res. 17*: 9909-9932).

6. Platforms with other recombinase system sites

A "double *lox*" targeting strategy mediated by Cre-recombinase (Bethke *et al.* (1997) *Nucl. Acids Res. 25*:2828-2834) can be used. This strategy employs a pair of heterospecific *lox* sites—*lox*A and *lox*B, which differ by one nucleotide in the 8 bp spacer region. Both sites are engineered into the artificial chromosome and also onto the targeting DNA vector. This allows for a direct site-specific insertion of a commercially relevant gene or genes by a Cre-catalyzed double crossover event. In essence a platform *ACes* is engineered with a hygromycin-resistance gene flanked by the double *lox* sites generating *lox-ACes,* which is maintained

in the thymidine kinase deficient cell, LMtk(-). The gene of interest, for example, for testing purposes, the green fluorescence protein gene, GFP and a HSV thymidine kinase gene (tk) marker, are engineered between the appropriate *lox* sites of the targeting vector. The vector DNA is cotransfected with plasmid pBS185 (Life Technologies) encoding the *Cre* recombinase gene into mammalian cells maintaining the dual-*lox* artificial chromosome. Transient expression of the *Cre* recombinase catalyzes the site-specific insertion of the gene and the tk-gene onto the artificial chromosome. The transfected cells are grown in HAT medium that selects for only those cells that have integrated and expressed the thymidine kinase gene. The HAT^R colonies are screened by PCR analyses to identify artificial chromosomes with the desired insertion.

To generate the *lox-ACes*, Lambda-Hyg^R-*lox* DNA is transfected into the LMtk(-) cell line harboring the precursor *ACes*. Hygromycin-resistant colonies are analyzed by FISH and Southern blotting for the presence of a single copy insert on the *ACes*.

To demonstrate the gene replacement technology, cell lines containing candidate *lox-ACes* are cotransfected with pTK-GFP-*lox* and pBS185 (encoding the *Cre* recombinase gene) DNA. After transfection, transient expression of plasmid pBS185 will provide sufficient burst of *Cre* recombinase activity to catalyze DNA recombination at the *lox* sites. Thus, a double crossover event between the *ACes* target and the exogenous targeting plasmid carrying the *lox*A and *lox*B permits the simple replacement of the hygromycin-resistance gene on the *lox-ACes* for the tk-GFP cassette from the targeting plasmid, with no integration of vector DNA. Transfected cells are grown in HAT-media to select for tk-expression. Correct targeting will result in the generation of HAT^R, hygromycin sensitive, and green fluorescent cells. The desired integration event is verified by Southern and PCR analyses. Specific PCR primer sets

are used to amplify DNA sequences flanking the individual *lox*A and *lox*B sites on the *lox-ACes* before and after homologous recombination.

D. Exemplary applications of the Platform *ACes*

Platform *ACes* are applicable and tractable for different/optimized cell lines. Those that include a fluorescent marker, for example, can be purified and isolated using fluorescent activated cell sorting (FACS), and subsequently delivered to a target cell. Those with selectable markers provide for efficient selection and provide a growth advantage. Platform *ACes* allow multiple payload delivery of donor target vectors via a positive-selection site-specific, recombination system, and they allow for the inclusion of additional genetic factors that improve protein production and protein quality.

The construction and use of the platform *ACes* as provided for each application may be similarly applied to other applications. Particular descriptions are for exemplification.

1. Cellular Protein Production Platform *ACes* (CPP *ACes*)

As described herein, *ACes* can be produced from acrocentric chromosomes in rodent (mouse, hamster) cell lines via megareplicator induced amplification of heterochromatin/rDNA sequences. Such *ACes* are ideal for cellular protein production as well as other applications described herein and known to those of skill in the art. *ACes* platforms that contain a plurality of recombination sites are particularly suitable for engineering as cellular protein production systems.

In one embodiment, CPP *ACes* involve a two-component system: the platform chromosome containing multiple engineering sites and the donor target vector containing a platform-specific recombination site with designed expression cassettes (see Figure 9).

The platform *ACes* can be produced from any artificial chromosome, particularly the amplification-based artificial chromosomes.

For exemplification, they are produced from rodent artificial chromosomes produced from acrocentric chromosomes using the technology of U.S. Patent Nos. 6,077,697 and 6,025,155 and published International PCT application No. WO 97/40183, in which nucleic acid is targeted to the pericentric heterochromatic, and, particularly into rDNA to initiate the replication event(s). The *ACes* can be produced directly in the chosen cellular protein production cell lines, such as, but not limited to, CHO cells, hybridomas, plant cells, plant tissues, plant protoplasts, stem cells and plant calli.

a. Platform Construction

In the exemplary embodiment, the initial *de novo* platform construction requires co-transfecting with excess targeting DNA, such as, rDNA or lambda DNA without an *attP* region, and an engineered selectable marker. The engineered selectable marker should contain promoter, generally a constitutive promoter, such as human, viral, i.e., adenovirus or SV40 promoter, including the human ferritin heavy chain promoter (SEQ ID NO:128), SV40 and EF1α promoters, to control expression of a marker gene that provides a selective growth advantage to the cell. An example of such a marker gene is the *E. coli hisD* gene (encoding histidinol dehydrogenase) which is homologous and analogous to the *S. typhimurium hisD* a dominant marker selection system for mammalian cells previously described (see, Hartman *et al.* (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*:8047-8051). Since histidine is an essential amino acid in mammals and a nutritional requirement in cell culture, the *E. coli hisD* gene can be used to select for histidine prototrophy in defined media. Furthermore more stringent selection can be placed on the cells by including histinol in the medium. Histidinol is itself permeable and toxic to cells. The *hisD* provides a means of detoxification.

Placed between the promoter and the marker gene is the bacteriophage lambda *attP* site to use the bacteriophage lambda integrase dependent site-specific recombination system (described herein). The insertion of an *attP* site downstream of a promoter element provide forward selection of site-specific recombination events onto the platform *ACes*.

b. Donor Target Vector Construction

A second component of the CPP platform *ACes* system involves the construction of donor target vectors containing a gene product(s) of interest for the CPP platform *ACes*. Individual donor target vectors can be designed for each gene product to be expressed thus enabling maximum usage of a *de novo* constructed platform *ACes*, so that one or a few CPP platform *ACes* will be required for many gene targets.

A key feature of the donor vector target is the *promoterless* marker gene containing an upstream *attB* site (marker 2 on figure 9). Normally the marker would not be expressed unless it is placed downstream of a promoter sequence. As discussed above, using the lambda integrase technology (λINT_{E174R} on Figure 8 and Figure 9), site-specific recombination between the *attB* site on the vector and the promoter-*attP* site on the CPP platform *ACes* result in the expression of the donor target vector marker providing positive selection for the site-specific event. Site-specific recombination events on the CPP *ACes* versus random integrations next to a promoter in the genome (false positive) can be quickly screened by designing primers to detect the correct event by PCR. In addition, since the lambda integrase reaction is unidirectional, i.e. excision reaction is not possible, a number of unique targets can be loaded onto the CPP platform *ACes* limited only by the number of markers available.

Additional features of the donor target vector include gene target expression cassettes flanked by either chromatin insulator regions, matrix attachment regions (MAR) or scaffold attachment regions (SAR). The use of these regions will provide a more "open" chromatin environment for gene expression and help alleviate silencing. An example of such a cassette for expressing a monoclonal antibody is described. For this purpose, a strong constitutive promoter, e.g. chicken β-actin or RNA PolI, is used to drive the expression of the heavy and light chain open reading frames. The heavy and light chain sequences flank a nonattenuated human IRES ($IRES_H$; from the 5'UTR of NRF1 gene; see Oumard et al., 2000, *Mol. and Cell Biol.*, 20(8):2755-2759) element thereby coordinating transcription of both heavy and light chain sequence. Distal to the light chain open reading frame resides an additional viral encoded IRES ($IRES_V$ modified ECMV internal ribosomal entry site (IRES)) element attenuating the expression of the fluorescent marker gene hrGFP from *Renilla* (Stratagene). By linking the hrGFP with an attenuated IRES, the heavy and light chains along with the hrGFP are monocistronic. Thus, the identification of hrGFP fluorescing cells will provide a means to detect protein producing cells. In addition, high producing cell lines can be identified and isolated by FACS thereby decreasing the time frame in finding high expressers. Functional monoclonal antibody will be confirmed by ELISA.

c. Additional components in cellular protein production platform *ACes* (CPP *Aces*)

In addition to the aforementioned CPP *ACes* system, other genetic factors can be included to enhance the yield and quality of the expressed protein. Again to provide maximum flexibility, these additional factors can be inserted onto the CPP platform *ACes* by λINTE174R dependent site-specific recombination. Other factors that could be used with a CPP

Platform *ACes* include for example, adenovirus E1a transactivation system which upregulates both cellular and viral promoters (see, e.g., Svensson and Akusjarvi (1984) EMBO 3:789-794; and US patents 5,866,359; 4,775,630 and 4,920,211).

d. Targets for CHO-*ACes* engineering to enhance cell growth, such as CHO cell growth and protein production/ quality

If adding these additional factors onto the CPP *ACes* is not prudent or desired, the host cell, CHO cells, can be engineered to express these factors (see, below, targets for CHO-*ACes* engineering to enhance CHO cell growth and protein production/quality). Additional factors to consider including are addition of insulin or IGF-1 to sustain viabililty; human sialyltransferases or related factors to produce more human-like glycoproteins; expression of factors to decrease ammonium accumulation during cell growth; expression of factors to inhibit apoptosis; expression of factors to improve protein secretion and protein folding; and expression of factors to permit serum-free transfection and selection.

1) Addition of insulin or IGF-1 to sustain viabililty

Stimulatory factors and/or their receptors are expressed to set up an autocrine loop, to improve cell growth, such as CHO cell growth. Two exemplary candidates are insulin and IGF-1 (see, Biotechnol Prog 2000 Sep;16(5):693-7). Insulin is the most commonly used growth factor for sustaining cell growth and viability in serum-free Chinese hamster ovary (CHO) cell cultures. Insulin and IGF-1 analog (LongR(3) serve as growth and viability factors for CHO cells.

CHO cells were modified to produce higher levels of essential nutrients and factors. A serum-free (SF) medium for dihydrofolate reductase-deficient Chinese hamster ovary cells (DG44 cells) was prepared. Chinese hamster ovary cells (DG44 cells), which are normally

maintained in 10% serum medium, were gradually weaned to 0.5% serum medium to increase the probability of successful growth in SF medium (see, Kim *et al.* (199) *In Vitro Cell Dev Biol Anim 35(4)*:178-82). A SF medium (SF-DG44) was formulated by supplementing the basal medium with these components; basal medium was prepared by supplementing Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 with hypoxanthine (10 mg/l) and thymidine (10 mg/l). Development of a SF medium for DG44 cells was facilitated using a Plackett-Burman design technique and weaning of cells.

2) Human sialyltransferases or related factors to produce more human-like glycoproteins

CHO cells have been modified by increasing their ability to process protein via addition of complex carbohydrates. This has been achieved by overexpression of relevant processing enzymes, or in some cases, reducing expression of relevant enzymes (see, Bragonzi *et al.* (2000) *Biochim Biophys Acta 1474(3)*:273-282; see, also Weikert *et al.* (1999) *Nature biotech. 17*:1116-11121; Ferrari J *et al.* (1998) *Biotechnol Bioeng 60(5)*:589-95). A CHO cell line expressing alpha2,6-sialyltransferase was developed for the production of human-like sialylated recombinant glycoproteins. The sialylation defect of CHO cells can be corrected by transfecting the alpha2,6-sialyltransferase (alpha2,6-ST) cDNA into the cells. Glycoproteins produced by such CHO cells display alpha2,6-and alpha2,3-linked terminal sialic acid residues, similar to human glycoproteins.

As another example for improving the production of human-like sialylated recombinant glycoproteins, a CHO cell line has been developed that constitutively expresses sialidase antisense RNA (see, Ferrari J *et al.* (1998) *Biotechnol Bioeng 60(5)*:589-95). Several antisense expression

vectors were prepared using different regions of the sialidase gene. Co-transfection of the antisense constructs with a vector conferring puromycin resistance gave rise to over 40 puromycin resistant clones that were screened for sialidase activity. A 5′ 474 bp coding segment of the sialidase cDNA, in the inverted orientation in an SV 40-based expression vector, gave maximal reduction of the sialidase activity to about 40% wild-type values.

Oligosaccharide biosynthesis pathways in mammalian cells have been engineered for generation of recombinant glycoproteins (see, *e.g.*, Sburlati (1998) *Biotechnol Prog 14(2)*:189-92), which describes a Chinese hamster ovary (CHO) cell line capable of producing bisected oligosaccharides on glycoproteins. This cell line was created by overexpression of a recombinant N-acetylglucosaminyltransferase III (GnT-III) (see, also, Prati *et al.* (1998) *Biotechnol Bioeng 59(4)*:445-50, which describes antisense strategies for glycosylation engineering of CHO cells).

3) Expression of factors to decrease ammonium accumulation during cell growth

Excess ammonium, which is a by-product of CHO cell metabolism can have detrimental effects on cell growth and protein quality (see, Yang *et al.* (2000) *Biotechnol Bioeng 68(4)*:370-80). To solve this problem ammonium levels were modified by overexpressing carbamoyl phosphate synthetase I and ornithine transcarbamoylase or glutamine synthetase in CHO cells. Such modification resulted in reduced ammonium levels observed and an increase in the growth rate (see Kim *et al.* (2000) *J Biotechnol 81(2-3)*:129-40; and Enosawa *et al.* (1997) *Cell Transplant 6(5)*:537-40).

4) Expression of factors to improve protein secretion and protein folding

Overexpression of relevant enzymes can be engineered into the *ACes* to improve protein secretion and folding.

5) Expression of factors to permit serum-free transfection and selection

It is advantageous to have the ability to convert CHO cells in suspension growing in serum free medium to adherence with out having to resort to serum addition. Laminin or fibronectin addition is sufficient to make cells adherent (see, *e.g.*, Zaworski *et al.* (1993) *Biotechniques 15(5)*:863-6) so that expressing either of these genes in CHO cells under an inducible promoter should allow for reversible shift to adherence without requiring serum addition.

2. Platform *ACes* and Gene Therapy

The platform *ACes* provided herein are contemplated for use in mammalian gene therapy, particularly human gene therapy. Human *ACes* can be derived from human acrocentric chromosomes from human host cells, in which the amplified sequences are heterochromatic and/or human rDNA. Different platform *ACes* applicable for different tissue cell types are provided. The *ACes* for gene therapy can contain a single copy of a therapeutic gene inserted into a defined location on platform *ACes*. Therapeutic genes include genomic clones, cDNA, hybrid genes and other combinations of sequences. Preferred selectable markers are those from the mammalian host, such as human derived factors so that they are non-immunogenic, non-toxic and allow for efficient selection, such as by FACS and/or drug resistance.

Platform *ACes*, useful for gene therapy and other applications, as noted herein, can be generated by megareplicator dependent amplification, such as by the methods in U.S. Patent Nos. 6,077,697 and 6,025,155 and published International PCT application No. WO 97/40183. In one embodiment, human *ACes* are produced using

human rDNA constructs that target rDNA arrays on human acrocentric chromosomes and induce the megareplicator in human cells, particularly in primary cell lines (with sufficient number of doublings to form the *ACes*) or stem cells (such as hematopoietic stem cells, mesenchymal stem cells, adult stem cells or embryonic stem cells) to avoid the introduction of potentially harmful rearranged DNA sequences present in many transformed cell lines. Megareplicator induced *ACes* formation can result in multiple copies of targeting DNA/selectable markers in each amplification block on both chromosomal arms of the platform *ACes*.

In view of the considerations regarding immunogenicity and toxicity, the production of human platform *ACes* for gene therapy applications employs a two component system analogous to the platform *ACes* designed for cellular protein production (CPP platform *ACes*). The system includes a platform chromosome of entirely human DNA origin containing multiple engineering sites and a gene target vector carrying the therapeutic gene of interest.

a. Platform Construction

The initial *de novo* construction of the platform chromosome employs the co-transfection of excess targeting DNA and a selectable marker. In one embodiment, the DNA is targeted to the rDNA arrays on the human acrocentric chromosomes (chromosomes 13, 14, 15, 21 and 22). For example, two large human rDNA containing PAC clones 18714 and 18720 and the human PAC clone 558F8 are used for targeting (Genome Research (ML) now Incyte, BACPAC Resources, 747 52nd Street, Oakland CA). The mouse rDNA clone pFK161 (SEQ ID NO: 118), which was used to make the human SATAC from the 94-3 hamster/human hybrid cell line (see, *e.g.*, published International PCT application No. WO 97/40183 and Csonka, *et al, Journal of Cell Science*

113:3207-32161 and Example 1 for a description of pFK161) can also be used.

For animal applications, selectable markers should be non-immunogenic in the animal, such as a human, and include, but are not limited to: human nerve growth factor receptor (detected with a MAb, such as described in US patent 6,365,373); truncated human growth factor receptor (detected with MAb), mutant human dihyrofolate reductase (DHFR; fluorescent MTX substrate available); secreted alkaline phosphatase (SEAP; fluorescent substrate available); human thymidylate synthase (TS; confers resistance to anti-cancer agent fluorodeoxyuridine); human glutathione S-transferase alpha (GSTA1; conjugates glutathione to the stem cell selective alkylator busulfan; chemoprotective selectable marker in CD34+ cells); CD24 cell surface antigen in hematopoietic stem cells; human CAD gene to confer resistance to N-phosphonacetyl-L-aspartate (PALA); human multi-drug resistance-1 (MDR-1; P-glycoprotein surface protein selectable by increased drug resistance or enriched by FACS); human CD25 (IL-2α; detectable by Mab-FITC); Methylguanine-DNA methyltransferase (MGMT; selectable by carmustine); and Cytidine deaminase (CD; selectable by Ara-C).

Since megareplicator induced amplification generates multiple copies of the selectable marker, a second consideration for the selection of the human marker is the resulting dose of the expressed marker after *ACes* formation. High level of expression of certain markers may be detrimental to the cell and/or result in autoimmunity. One method to decrease the dose of the marker protein is by shortening its half-life, such as via the fusion of the well-conserved human ubiquitin tag (a 76 amino acid sequence) thus leading to increased turnover of the selectable marker. This has been used successfully for a number of reporter

systems including DHFR (see, *e.g.*, Stack *et al.* (2000) *Nature Biotechnology 18*:1298-1302 and references cited therein).

Using the ubiquitin tagged protein, a human selectable marker system analogous to the CPP *ACes* described herein is constructed. Briefly, a tagged selectable marker, such as for example one of those described herein, is cloned downstream of an *attP* site and expressed from a human promoter. Exemplary promoters contemplated for use herein include, but are not limited to, the human ferritin heavy chain promoter (SEQ ID NO:128); RNA PolI; EF1α; TR; glyceraldehyde-3-phosphate dehydrogenase core promoter (GAP); a GAP core promoter including a proximal insulin inducible element the intervening GAP sequence; phosphofructokinase promoter; and phosphoglycerate kinase promoter. Also contemplated herein is an aldolase A promoter H1 & H2 (representing closely spaced transcriptional start sites) along with the proximal H enhancer. There are 4 promoters (e.g., transcriptional start sites) for this gene, each having different regulatory and tissue activity. The H (most proximal 2) promoters are ubiquitously expressed off the H enhancer. This resulting marker can then be co-transfected along with excess human rDNA targeting sequence into the host cells. An important criteria for the selection of the

recipient cells is sufficient number of cell doublings for the formation and detection of *ACes*. Accordingly, the co-transfections should be attempted in human primary cells that can be cultured for long periods of time, such as for example stem cells (e.g., hematopoietic, mesenchymal, adult or embryonic stem cells), or the like. Additional cell types, include, but are not limited to: single gene transfected cells exhibiting increased life-span; over-expressing c-myc cells, e.g. MSU1.1 (Morgan et al., 1991, Exp. Cell Res., Nov;197(1):125-136); over-expressing telomerase lines,

such as TERT cells; SV40 large T-antigen transfected lines; tumor cell lines, such as HT1080; and hybrid human cell lines, such as the 94-3 hamster/human hybrid cell line.

b. Gene Target Vector

The second component of the GT platform *ACes* (GT *ACes*) system involves the use of engineered target vectors carrying the therapeutic gene of interest. These are introduced onto the GT platform *ACes* via site-specific recombination. As with the CPP *ACes*, the use of engineered target vectors maximizes the use of the *de novo* generated GT platform *ACes* for most gene targets. Furthermore, using lambda integrase technology, GT platform *ACes* containing multiple *attP* sites permits the opportunity to incorporate multiple therapeutic targets onto a single platform. This could be of value in cases where a defined therapy requires multiple gene targets, a single therapeutic target requires an additional gene regulatory factor or a GT *ACes* requires a "kill" switch.

Similar to the CPP *ACes*, a feature of the gene target vector is the *promoterless* marker gene containing an upstream *attB* site (marker 2 on Figure 9). Normally, the marker (in this case, a cell surface antigen that can be sorted by FACS would be ideal) would not be expressed unless it is placed downstream of a promoter sequence. Using the lambda integrase technology (λINT_{E174R} on figure 9), site-specific recombination between the *attB* site on the vector and the promoter- *attP* site on the GT platform *ACes* results in the expression of marker#2 on the gene target vector, i.e. positive selection for the site-specific event. Site-specific recombination events on the GT *ACes* versus random integrations next to a promoter in the genome (false positive) can be quickly screened by designing primers to detect the correct event by PCR.

For expression of the therapeutic gene, human specific promoters, such as a ferritin heavy chain promoter (SEQ ID NO:128); EF1α or RNA

PolI, are used. These promoters are for high level expression of a cDNA encoded therapeutic protein. In addition to expressing cDNA (or even hybrid cDNA/artificial intron constructs), the GT platform *ACes* are used for engineering and expressing large genomic fragments carrying therapeutic genes of interest expressed from native promoter sequences. This is of importance in situations where the therapy requires precise cell specific expression or in instances where expression is best achieved from genomic clones versus cDNA.

3. Selectable markers for use, for example, in Gene Therapy (GT)

The following are selectable markers that can be incorporated into human *ACes* and used for selection.

Dual Resistance to 4-Hydroperoxycyclophosphamide and Methotrexate by Retroviral Transfer of the Human Aldehyde Dehydrogenase Class 1 Gene and a Mutated Dihydrofolate Reductase Gene

The genetic transfer of drug resistance to hematopoietic cells is one approach to overcoming myelosuppression caused by high-dose chemotherapy. Because cyclophosphamide (CTX) and methotrexate (MTX) are commonly used non-cross-resistant drugs, generation of dual drug resistance in hematopoietic cells that allows dose intensification may increase anti-tumor effects and circumvent the emergence of drug-resistant tumors, a retroviral vector containing a human cytosolic ALDH-1-encoding DNA clone and a human doubly mutated DHFR-encoding clone (Phe22/Ser31; termed F/S in the description of constructs) to generate increased resistance to CTX and MTX were constructed (Takebe *et al.* (2001) *Mol Ther 3(1)*:88-96). This construct may be useful for protecting patients from high-dose CTX- and MTX-induced myelosuppression. *ACes* can be similarly constructed.

Multiple mechanisms of N-phosphonacetyl-L-aspartate resistance in human cell lines: carbamyl-P synthetase/aspartate transcarbamylase/dihydro-orotase gene amplification is frequent only when chromosome 2 is rearranged

Rodent cells resistant to N-phosphonacetyl-L-aspartate (PALA) invariably contain amplified carbamyl-P synthetase/aspartate transcarbamylase/dihydro-orotase (CAD) genes, usually in widely spaced tandem arrays present as extensions of the same chromosome arm that carries a single copy of CAD in normal cells (Smith *et al.* (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*:1816-21). In contrast, amplification of CAD is very infrequent in several human tumor cell lines. Cell lines with minimal chromosomal rearrangement and with unrearranged copies of chromosome 2 rarely develop intrachromosomal amplifications of CAD. These cells frequently become resistant to PALA through a mechanism that increases the aspartate transcarbamylase activity with no increase in CAD copy number, or they obtain one extra copy of CAD by forming an isochromosome 2p or by retaining an extra copy of chromosome 2. In cells with multiple chromosomal aberrations and rearranged copies of chromosome 2, amplification of CAD as tandem arrays from rearranged chromosomes is the most frequent mechanism of PALA resistance. All of these different mechanisms of PALA resistance are blocked in normal human fibroblasts. Thus, *ACes* with multiple copies of the CAD gene would provide PALA resistance.

Retroviral coexpression of thymidylate synthase and dihydrofolate reductase confers fluoropyrimidine and antifolate resistance

Retroviral gene transfer of dominant selectable markers into hematopoietic cells can be used to select genetically modified cells in vivo or to attenuate the toxic effects of chemotherapeutic agents. Fantz *et al.* ((1998) *Biochem Biophys Res Comm 243(1)*:6-12) have shown that

retroviral gene transfer of thymidylate synthase (TS) confers resistance to TS directed anticancer agents and that co-expression of TS and dihydrofolate reductase (DHFR) confers resistance to TS and DHFR cytotoxic agents. Retroviral vectors encoding *Escherichia coli* TS, human TS, and the Tyr-to-His at residue 33 variant of human TS (Y33HhTS) were constructed and fibroblasts transfected with these vectors conferred comparable resistance to the TS-directed agent fluorodeoxyuridine (FdUrd, approximately 4-fold). Retroviral vectors that encode dual expression of Y33HhTS and the human L22Y DHFR (L22YhDHFR) variants conferred resistance to FdUrd (3- to 5-fold) and trimetrexate (30- to 140-fold). A L22YhDHFR-Y33HhTS chimeric retroviral vector was also constructed and transduced cells were resistant to FdUrd (3-fold), AG337 (3-fold), trimetrexate (100-fold) and methotrexate (5-fold). These results show that recombinant retroviruses can be used to transfer the cDNA that encodes TS and DHFR and dual expression in transduced cells is sufficiently high to confer resistance to TS and DHFR directed anticancer agents. *ACes* can be similarly constructed.

Human CD34+ cells do not express glutathione S-transferases alpha

The expression of glutathione S-transferases alpha (GST alpha) in human hematopoietic CD34+ cells and bone marrow was studied using RT-PCR and immunoblotting (Czerwinski M, Kiem *et al.* (1997) *Gene Ther 4(3)*:268-70). The GSTA1 protein conjugates glutathione to the stem cell selective alkylator busulfan. This reaction is the major pathway of elimination of the compound from the human body. Human hematopoietic CD34+ cells and bone marrow do not express GSTA1 message, which was present at a high level in liver, an organ relatively resistant to busulfan toxicity in comparison to bone marrow. Similarly, baboon CD34+ cells and dog bone marrow do not express GSTA1. Thus, human

GSTA1 is a chemoprotective selectable marker in human stem cell gene therapy and could be employed in *ACes* construction.

Selection of retrovirally transduced hematopoietic cells using CD24 as a marker of gene transfer

Pawliuk *et al.* ((1994) *Blood 84(9)*:2868-2877) have investigated the use of a cell surface antigen as a dominant selectable marker to facilitate the detection and selection of retrovirally infected target cells. The small coding region of the human cell surface antigen CD24 (approximately 240 bp) was introduced into a myeloproliferative sarcoma virus (MPSV)-based retroviral vector, which was then used to infect day 4 5-fluorouracil (5-FU)-treated murine bone marrow cells. Within 48 hours of termination of the infection procedure CD24-expressing cells were selected by fluorescent-activated cell sorting (FACS) with an antibody directed against the CD24 antigen. Functional analysis of these cells showed that they included not only in vitro clonogenic progenitors and day 12 colony-forming unit-spleen but also cells capable of competitive long-term hematopoietic repopulation. Double-antibody labeling studies performed on recipients of retrovirally transduced marrow cells showed that some granulocytes, macrophages, erythrocytes, and, to a lesser extent, B and T lymphocytes still expressed the transduced CD24 gene at high levels 4 months later. No gross abnormalities in hematopoiesis were detected in mice repopulated with CD24-expressing cells. These results show that the use of the CD24 cell surface antigen as a retrovirally encoded marker permits rapid, efficient, and nontoxic selection *in vitro* of infected primary cells, facilitates tracking and phenotyping of their progeny, and provides a tool to identify elements that regulate the expression of transduced genes in the most primitive hematopoietic cells. *ACes* could be similarly constructed.

DeltahGHR, a biosafe cell surface-labeling molecule for analysis and selection of genetically transduced human cells

A selectable marker for retroviral transduction and selection of human and murine cells is known (see, Garcia-Ortiz *et al.* (2000) *Hum Gene Ther 11(2)*:333-46). The molecule expressed on the cell surface of the transduced population is a truncated version of human growth hormone receptor (deltahGHR), capable of ligand (hGH) binding, but devoid of the domains involved in signal triggering. The engineered molecule is stably expressed in the target cells as an inert protein unable to trigger proliferation or to rescue the cells from apoptosis after ligand binding. This new marker, has a wide application spectrum, since hGHR in the human adult is highly expressed only in liver cells, and lower levels have been reported in certain lymphocyte cell populations. The deltahGHR label has high biosafety potential, as it belongs to a well-characterized hormonal system that is nonessential in adults, and there is extensive clinical experience with hGH administration in humans. The differential binding properties of several monoclonal antibodies (MAbs) are used in a cell rescue method in which the antibody used to select deltahGHR-transduced cells is eluted by competition with hGH or, alternatively biotinylated hGH is used to capture tagged cells. In the latter system, the final purified population is recovered free of attached antibodies in hGH (a substance approved for human use)-containing medium. Such a system could be used to identify *ACes* containing cells.

4. Transgenic models for evaluation of genes and discovery of new traits in plants

Of interest is the use of plants and plant cells containing artificial chromosomes for the evaluation of new genetic combinations and discovery of new traits. Artificial chromosomes, by virtue of the fact that they can contain significant amounts of DNA can also therefore encode

numerous genes and accordingly a multiplicity of traits. It is contemplated here that artificial chromosomes, when formed from one plant species, can be evaluated in a second plant species. The resultant phenotypic changes observed, for example, can indicate the nature of the genes contained within the DNA contained within the artificial chromosome, and hence permit the identification of novel genetic activities. Artificial chromosomes containing euchromatic DNA or partially containing euchromatic DNA can serve as a valuable source of new traits when transferred to an alien plant cell environment. For example, it is contemplated that artificial chromosomes derived from dicot plant species can be introduced into monocot plant species by transferring a dicot artificial chromosome. The dicot artificial chromosome possessing a region of euchromatic DNA containing expressed genes.

The artificial chromosomes can be designed to allow the artificial chromosome to recombine with the naturally occurring plant DNA in such a fashion that a large region of naturally occurring plant DNA becomes incorporated into the artificial chromosome. This allows the artificial chromosome to contain new genetic activities and hence carry novel traits. For example, an artificial chromosome can be introduced into a wild relative of a crop plant under conditions whereby a portion of the DNA present in the chromosomes of the wild relative is transferred to the artificial chromosome. After isolation of the artificial chromosome, this naturally occurring region of DNA from the wild relative, now located on the artificial chromosome can be introduced into the domesticated crop species and the genes encoded within the transferred DNA expressed and evaluated for utility. New traits and gene systems can be discovered in this fashion. The artificial chromosome can be modified to contain sequences that promote homologous recombination within plant cells, or

be modified to contain a genetic system that functions as a site-specific recombination system.

Artificial chromosomes modified to recombine with plant DNA offer many advantages for the discovery and evaluation of traits in different plant species. When the artificial chromosome containing DNA from one plant species is introduced into a new plant species, new traits and genes can be introduced. This use of an artificial chromosome allows for the ability to overcome the sexual barrier that prevents transfer of genes from one plant species to another species. Using artificial chromosomes in this fashion allows for many potentially valuable traits to be identified including traits that are typically found in wild species. Other valuable applications for artificial chromosomes include the ability to transfer large regions of DNA from one plant species to another, such as DNA encoding potentially valuable traits such as altered oil, carbohydrate or protein composition, multiple genes encoding enzymes capable of producing valuable plant secondary metabolites, genetic systems encoding valuable agronomic traits such as disease and insect resistance, genes encoding functions that allow association with soil bacterium such as growth promoting bacteria or nitrogen fixing bacteria, or genes encoding traits that confer freezing, drought or other stress tolerances. In this fashion, artificial chromosomes can be used to discover regions of plant DNA that encode valuable traits.

The artificial chromosome can also be designed to allow the transfer and subsequent incorporation of these valuable traits now located on the artificial chromosome into the natural chromosomes of a plant species. In this fashion the artificial chromosomes can be used to transfer large regions of DNA encoding traits normally found in one plant species into another plant species. In this fashion, it is possible to derive a plant cell that no longer needs to carry an artificial chromosome to

posses the novel trait. Thus, the artificial chromosome would serve as the transfer mechanism to permit the formation of plants with greater degree of genetic diversity.

The design of an artificial chromosome to accomplish the aforementioned purposes can include within the artificial chromosome the presence of specific DNA sequences capable of acting as sites for homologous recombination to take place. For example, the DNA sequence of *Arabidopsis* is now known. To construct an artificial chromosome capable of recombining with a specific region of *Arabidopsis* DNA, a sequence of *Arabidopsis* DNA, normally located near a chromosomal location encoding genes of potential interest can be introduced into an artificial chromosome by methods provided herein. It may be desirable to include a second region of DNA within the artificial chromosome that provides a second flanking sequence to the region encoding genes of potential interest, to promote a double recombination event which would ensure transfer of the entire chromosomal region, encoding genes of potential interest, to the artificial chromosome. The modified artificial chromosome, containing the DNA sequences capable of homologous recombination region, can then be introduced into *Arabidopsis* cells and the homologous recombination event selected.

It is convenient to include a marker gene to allow for the selection of a homologous recombination event. The marker gene is preferably inactive unless activated by an appropriate homologous recombination event. For example, US 5,272,071, describes a method where an inactive plant gene is activated by a recombination event such that desired homologous recombination events can be easily scored. Similarly, US 5,501,967 describes a method for the selection of homologous recombination events by activation of a silent selection gene first introduced into the plant DNA, the gene being activated by an appropriate

homologous recombination event. Both of these methods can be applied to enable a selective process to be included to select for recombination between an artificial chromosome and a plant chromosome. Once the homologous recombination event is detected, the artificial chromosome, once selected, is isolated and introduced into a recipient cell, for example, tobacco, corn, wheat or rice, and the expression of the newly introduced DNA sequences evaluated.

Phenotypic changes in the recipient plant cells containing the artificial chromosome, or in regenerated plants containing the artificial chromosome, allows for the evaluation of the nature of the traits encoded by the *Arabidopsis* DNA, under conditions naturally found in plant cells, including the naturally occurring arrangement of DNA sequences responsible for the developmental control of the traits in the normal chromosomal environment.

Traits such as durable fungal or bacterial disease resistance, new oil and carbohydrate compositions, valuable secondary metabolites such as phytosterols, flavonoids, efficient nitrogen fixation or mineral utilization, resistance to extremes of drought, heat or cold are all found within different populations of plant species and are often governed by multiple genes. The use of single gene transformation technologies does not permit the evaluation of the multiplicity of genes controlling many valuable traits. Thus, incorporation of these genes into artificial chromosomes allows the rapid evaluation of the utility of these genetic combinations in heterologous plant species.

The large scale order and structure of the artificial chromosome provides a number of unique advantages in screening for new utilities or novel phenotypes within heterologous plant species. The size of new DNA that can be carried by an artificial chromosome can be millions of base pairs of DNA, representing potentially numerous genes that may

have novel utility in a heterologous plant cell. The artificial chromosome is a "natural" environment for gene expression, the problems of variable gene expression and silencing seen for genes transferred by random insertion into a genome should not be observed. Similarly, there is no need to engineer the genes for expression, and the genes inserted would not need to be recombinant genes. Thus, one expects the expression from the transferred genes to be temporal and spatial, as observed in the species from where the genes were initially isolated. A valuable feature for these utilities is the ability to isolate the artificial chromosomes and to further isolate, manipulate and introduce into other cells artificial chromosomes carrying unique genetic compositions.

Thus, the use of artificial chromosomes and homologous recombination in plant cells can be used to isolate and identify many valuable crop traits.

In addition to the use of artificial chromosomes for the isolation and testing of large regions of naturally occurring DNA, methods for the use of artificial chromosomes and cloned DNA are also contemplated. Similar to that described above, artificial chromosomes can be used to carry large regions of cloned DNA, including that derived from other plant species.

The ability to incorporate novel DNA elements into an artificial chromosome as it is being formed allows for the development of artificial chromosomes specifically engineered as a platform for testing of new genetic combinations, or "genomic" discoveries for model species such as *Arabidopsis*. It is known that specific "recombinase" systems can be used in plant cells to excise or re-arrange genes. These same systems can be used to derive new gene combinations contained on an artificial chromosome.

The artificial chromosomes can be engineered as platforms to accept large regions of cloned DNA, such as that contained in Bacterial

Artificial Chromosomes (BACs) or Yeast Artificial Chromosomes (YACs). It is further contemplated, that as a result of the typical structure of artificial chromosomes containing tandemly repeated DNA blocks, that sequences other than cloned DNA sequence can be introduced by recombination processes. In particular recombination within a predefined region of the tandemly repeated DNA within the artificial chromosome provides a mechanism to "stack" numerous regions of cloned DNA, including large regions of DNA contained within BACs or YACs clones. Thus, multiple combinations of genes can be introduced onto artificial chromosomes and these combinations tested for functionality. In particular, it is contemplated that multiple YACs or BACs can be stacked onto an artificial chromosomes, the BACs or YACs containing multiple genes of complex pathways or multiple genetic pathways. The BACs or YACs are typically selected based on genetic information available within the public domain, for example from the Arabidopsis Information Management System (http://aims.cps.msu.edu/aims/index.html) or the information related to the plant DNA sequences available from the Institute for Genomic Research (http://www.tigr.org) and other sites known to those skilled in the art. Alternatively, clones can be chosen at random and evaluated for functionality. It is contemplated that combinations providing a desired phenotype can be identified by isolation of the artificial chromosome containing the combination and analyzing the nature of the inserted cloned DNA.

In this regard, it is contemplated that the use of site-specific recombination sequences can have considerable utility in developing artificial chromosomes containing DNA sequences recognized by recombinase enzymes and capable of accepting DNA sequences containing same. The use of site-specific recombination as a means to target an introduced DNA to a specific locus has been demonstrated in

the art and such methods can be employed. The recombinase systems can also be used to transfer the cloned DNA regions contained within the artificial chromosome to the naturally occurring plant or mammalian chromosomes.

As noted herein, many site-specific recombinases are known and can be identified (Kilby *et al.* (1993) *Trends in Genetics 9*:413-418). The three recombinase systems that have been extensively employed include: an activity identified as R encoded by the pSR1 plasmid of *Zygosaccharomyes rouxii*, FLP encoded for the 2um circular plasmid from *Saccharomyces cerevisiae* and *Cre-lox* from the phage P1.

The integration function of site-specific recombinases is contemplated as a means to assist in the derivation of genetic combinations on artificial chromosomes. In order to accomplish this, it is contemplated that a first step of introducing site-specific recombinase sites into the genome of a plant cell in an essentially random manner is conducted, such that the plant cell has one or more site-specific recombinase recognition sequences on one or more of the plant chromosomes. An artificial chromosome is then introduced into the plant cell, the artificial chromosome engineered to contain a recombinase recognition site (e.g., integration site) capable of being recognized by a site-specific recombinase. Optionally, a gene encoding a recombinase enzyme is also included, preferably under the control of an inducible promoter. Expression of the site-specific recombinase enzyme in the plant cell, either by induction of a inducible recombinase gene, or transient expression of a recombinase sequence, causes a site-specific recombination event to take place, leading to the insertion of a region of the plant chromosomal DNA (containing the recombinase recognition site) into the recombinase recognition site of the artificial chromosome, and forming an artificial chromosome containing plant chromosomal DNA.

The artificial chromosome can be isolated and introduced into a heterologous host, preferably a plant host, and expression of the newly introduced plant chromosomal DNA can be monitored and evaluated for desirable phenotypic changes. Accordingly, carrying out this recombination with a population of plant cells wherein the chromosomally located recombinase recognition site is randomly scattered throughout the chromosomes of the plant, can lead to the formation of a population of artificial chromosomes, each with a different region of plant chromosomal DNA, and each potentially representing a novel genetic combination.

This method requires the precise site-specific insertion of chromosomal DNA into the artificial chromosome. This precision has been demonstrated in the art. For example, Fukushige and Sauer ((1992) Proc. Natl. Acad. Sci. USA, 89:7905-7909) demonstrated that the *Cre-lox* homologous recombination system could be successfully employed to introduce DNA into a predefined locus in a chromosome of mammalian cells. In this demonstration a promoter-less antibiotic resistance gene modified to include a *lox* sequence at the 5′ end of the coding region was introduced into CHO cells. Cells were re-transformed by electroporation with a plasmid that contained a promoter with a *lox* sequence and a transiently expressed *Cre* recombinase gene. Under the conditions employed, the expression of the *Cre* enzyme catalyzed the homologous recombination between the *lox* site in the chromosomally located promoter-less antibiotic resistance gene, and the *lox* site in the introduced promoter sequence, leading to the formation of a functional antibiotic resistance gene. The authors demonstrated efficient and correct targeting of the introduced sequence, 54 of 56 lines analyzed corresponded to the predicted single copy insertion of the DNA due to *Cre* catalyzed site-specific homologous recombination between the *lox* sequences.

Accordingly a *lox* sequence may be first added to a genome of a plant species capable of being transformed and regenerated to a whole plant to serve as a recombinase target DNA sequence for recombination with an artificial chromosome. The *lox* sequence may be optimally modified to further contain a selectable marker which is inactive but can be activated by insertion of the *lox* recombinase recognition sequence into the artificial chromosome.

A promoterless marker gene or selectable marker gene linked to the recombinase recognition sequence, which is first inserted into the chromosomes of a plant cell can be used to engineer a platform chromosome. A promoter is linked to a recombinase recognition site, in an orientation that allows the promoter to control the expression of the marker or selectable marker gene upon recombination within the artificial chromosome. Upon a site-specific recombination event between a recombinase recognition site in a plant chromosome and the recombinase recognition site within the introduced artificial chromosome, a cell is derived with a recombined artificial chromosome, the artificial chromosome containing an active marker or selectable marker activity that permits the identification and or selection of the cell.

The artificial chromosomes can be transferred to other plant or animal species and the functionality of the new combinations tested. The ability to conduct such an inter-chromosomal transfer of sequences has been demonstrated in the art. For example, the use of the *Cre-lox* recombinase system to cause a chromosome recombination event between two chromatids of different chromosomes has been shown.

Any number of recombination systems may be employed as described herein, such as, but not limited to, bacterially derived systems such as the att/int system of phage lambda, and the Gin/gix system.

More than one recombination system may be employed, including, for example, one recombinase system for the introduction of DNA into an artificial chromosome, and a second recombinase system for the subsequent transfer of the newly introduced DNA contained within an artificial chromosome into the naturally occurring chromosome of a second plant species. The choice of the specific recombination system used will be dependent on the nature of the modification contemplated.

By having the ability to isolate an artificial chromosome, in particular, artificial chromosomes containing plant chromosomal DNA introduced via site-specific recombination, and re-introduce the chromosome into other mammalian or plant cells, particularly plant cells, these new combinations can be evaluated in different crop species without the need to first isolate and modify the genes, or carry out multiple transformations or gene transfers to achieve the same combination isolation and testing combinations of the genes in plants. The use of a site-specific recombinase also allows the convenient recovery of the plant chromosomal region into other recombinant DNA vectors and systems, such as mammalian or insect systems, for manipulation and study.

Also contemplated herein are *ACes*, cell lines and methods for use in screening a new chromosomal combinations, deletions, truncations with eucaryotic genome that take advantage of the site-specific recombination systems incorporated onto platform *ACes* provided herein. For example, provided herein is a cell line useful for making a library of *ACes*, comprising a multiplicity of heterologous recombination sites randomly integrated throughout the endogenous chromosomes. Also provided herein is a method of making a library of *ACes* comprising random portions of a genome, comprising introducing one or more *ACes* into a cell line comprising a multiplicity of heterologous recombination

sites randomly integrated throughout the endogenous chromosomes, under conditions that promote the site-specific chromosomal arm exchange of the *ACes* into, and out of, a multiplicity of the heterologous recombination sites within the cell's chromosomal DNA; and isolating said multiplicity of *ACes*, thereby producing a library of *ACes* whereby multiple *ACes* have different portions of the genome within. Also provided herein is a library of cells useful for genomic screening, said library comprising a multiplicity of cells, wherein each cell comprises an *ACes* having a mutually exclusive portion of a chromosomal nucleic acid therein. The library of cells can be from a different species and/or cell type than the chromosomal nucleic acid within the *ACes*. Also provided is a method of making one or more cell lines, comprising

a) integrating into endogenous chromosomal DNA of a selected cell species, a multiplicity of heterologous recombination sites,

b) introducing a multiplicity of *ACes* under conditions that promote the site-specific chromosomal arm exchange of the *ACes* into, and out of, a multiplicity of the heterologous recombination sites integrated within the cell's endogenous chromosomal DNA;

c) isolating said multiplicity of *ACes*, thereby producing a library of *ACes* whereby a multiplicity of *ACes* have mutually exclusive portions of the endogenous chromosomal DNA therein;

d) introducing the isolated multiplicity of *ACes* of step c) into a multiplicity of cells, thereby creating a library of cells;

e) selecting different cells having mutually exclusive *ACes* therein and clonally expanding or differentiating said different cells into clonal cell cultures, thereby creating one or more cell lines.

These *ACes*, cell lines and methods utilize the site-specific recombination sites on platform *ACes* analogous YAC manipulation related to: the methods of generating terminal deletions in normal and

artificial chromosomes (e.g., *ACes*; as described in Vollrath et al., 1988, *PNAS, USA*, 85:6027-66031; and Pavan et al., *PNAS, USA*, 87:1300-1304); the methods of generating interstitial deletions in normal and artificial chromosomes (as described in Campbell et al., 1991, *PNAS, USA*, 888:5744-5748); and the methods of detecting homologous recombination between two *ACes* (as described in Cellini et al., 1991, *Nuc. Acid Res.*, 19(5):997-1000).

5. Use of plateform *ACes* in Pharmacogenomic/toxicology applications (development of "Reporter *ACes*")

In addition to the placement of genes onto *ACes* chromosomes for therapeutic protein production or gene therapy, the platform can be engineered via the IntR lambda integrase to carry reporter-linked constructs (reporter genes) that monitor changes in cellular physiology as measured by the particular reporter gene (or a series of different reporter genes) readout. The reporter linked constructs are designed to include a gene that can be detected (by for example fluorescence, drug resistance, immunohistochemistry, or transcript production, and the like) with well-known regulatory sequences that would control the expression of the detectable gene. Exemplary regulatory promoter sequences are well-known in the art:

A) Reporter *ACes* for drug pathway screening

The *ACes* can be engineered to carry reporter-linked constructs that indicate a signal is being transduced through one or a number of pathways. For example, transcriptionally regulated promoters from genes at the end (or any other chosen point) of particular signal transduction pathways could be engineered on the *ACes* to express the appropriate readout (either by fluorescent protein production or drug resistance) when the pathway is activated (or down-regulated as well). In one embodiment, a number of reporters from different can be placed on a

ACes chromosome. Cells (and/or whole animals) containing such a Reporter *ACes* could be exposed to a variety of drugs or compounds and monitored for the effects of the drugs or compounds upon the selected pathway(s) by the reporter gene(s). Thus, drugs or compounds can be classified or identified by particular pathways they excite or down-regulate. Similarly, transcriptional profiles obtained from genomic array experiments can be biologically validated using the reporter *ACes* provided herein.

B) Reporter *ACes* for toxic compound testing

Environmental or man-made genotoxicants can be tested in cell lines carrying a number of reporter-genes platform *ACes* linked to promoters that are transcriptionally regulated in response to DNA damage, induced apoptosis or necrosis, and cell-cycle perturbations. Furthermore, new drugs and/or compounds could be tested in a similar manner with the genotoxicant *ACes* reporter for their cellular/genetic toxicity by such a screen. Likewise, toxic compound testing could be carried out in whole transgenic animals carrying the *ACes* chromosome that measures genotoxicant exposure ("canary in a coal mine"). Thus, the same or similar type *ACes* could be used for toxicity testing in either a cell-based or whole animal setting. An example would include *ACes* that carry reporter-linked genes controlled by various cytochrome P450 profiled promoters and the like.

C) Reporter *ACes* for individualized pharmacogenomics/drug profiling

A common disease may arise via various mechanisms. In many instances there are multiple treatments available for a given disease. However, the success of a given treatment may depend upon the mechanism by which the disease originated and/or by the genetic background of the patient. In order to establish the most effective

treatment for a given patient one could utilize the *ACes* reporters provided herein. *ACes* reporters can be used in patient cell samples to determine an individualized drug regimen for the patient. In addition, potential polymorphisms affecting the transcriptional regulation of an individual's particular gene can be assessed by this approach.

D) Reporter *ACes* for classification of similar patient tumors

As with other diseases as described in 5.C) above, cancer cells arise via different mechanisms. Furthermore, as a cancerous cell propagates it may undergo genomic alterations. An *ACes* reporter transferred to cells of different patients having the same disease, i.e. similar cancers, could be used to categorize the particular cancer of each patient, thereby facilitating the identification of the most effective therapeutic regimen. Examples would include the validation of array profiling of certain classes of breast cancers. Subsequently, appropriate drug profiling could be carried out as described above.

E) Reporter *ACes* as a "differentiation" sensor

Using the *ACes* reporter as a "differentiation" sensor in stem cells or other progenitor cells in order to enrich by selection (either FACS based screening, drug selection and/or use of suicide gene) for a particular class of differentiated or undifferentiated cells. For example, in one embodiment, this assay could also be used for compound screening for small molecule modifiers of cell differentiation.

F) Whole animal studies with Reporter *ACes*

Finally, with whole-body fluorescence imaging technology (Yang et al. (2000) PNAS 97:12278) any of the above Reporter *ACes* methods could be used in conjunction with whole-body imaging to monitor reporter genes within whole animals without sacrificing the animal. This would allow temporal and spatial analysis of expression patterns under a given set of conditions. The conditions tested may include for example, normal

differentiation of a stem cell, response to drug or compound treatment whether targeted to the diseased tissue or presented systemically, response to genotoxicants, and the like.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1

pFK161

Cosmid pFK161 (SEQ ID NO: 118) was obtained from Dr. Gyula Hadlaczky and contains a 9 kb *Not*I insert derived from a murine rDNA repeat (see clone 161 described in PCT Application Publication No. WO97/40183 by Hadlaczky *et al.* for a description of this cosmid). This cosmid, referred to as clone 161 contains sequence corresponding to nucleotides 10,232-15,000 in SEQ ID NO. 26. It was produced by inserting fragments of the megachromosome (see, U.S. Patent No. 6,077,697 and International PCT application No. WO 97/40183). For example, H1D3, which was deposited at the European Collection of Animal Cell Culture (ECACC) under Accession No. 96040929, is a mouse-hamster hybrid cell line carrying this megachromosome into plasmid pWE15 (Stratagene, La Jolla, California; SEQ ID No. 31) as follows. Half of a 100 μl low melting point agarose block (mega-plug) containing isolated SATACs was digested with *Not*I overnight at 37°C. Plasmid pWE15 was similarly digested with *Not*I overnight. The mega-plug was then melted and mixed with the digested plasmid, ligation buffer and T4 DNA ligase. Ligation was conducted at 16°C overnight. Bacterial DH5α cells were transformed with the ligation product and transformed cells were plated onto LB/Amp plates. Fifteen to twenty colonies were grown on each plate for a total of 189 colonies. Plasmid DNA was isolated from colonies that survived growth on LB/Amp medium and analyzed by Southern blot hybridization for the presence of DNA that

hybridized to a pUC19 probe. This screening methodology assured that all clones, even clones lacking an insert but yet containing the pWE15 plasmid, would be detected.

Liquid cultures of all 189 transformants were used to generate cosmid minipreps for analysis of restriction sites within the insert DNA. Six of the original 189 cosmid clones contained an insert. These clones were designated as follows: 28 (~9-kb insert), 30 (~ 9-kb insert), 60 (~4-kb insert), 113 (~9-kb insert), 157 (~9-kb insert) and 161 (~9-kb insert). Restriction enzyme analysis indicated that three of the clones (113, 157 and 161) contained the same insert.

For sequence analysis the insert of cosmid clone no. 161 was subcloned as follows. To obtain the end fragments of the insert of clone no. 161, the clone was digested with *Not*I and *Bam*HI and ligated with *Not*I/*Bam*HI-digested pBluescript KS (Stratagene, La Jolla, California). Two fragments of the insert of clone no. 161 were obtained: a 0.2-kb and a 0.7-kb insert fragment. To subclone the internal fragment of the insert of clone no. 161, the same digest was ligated with *Bam*HI-digested pUC19. Three fragments of the insert of clone no. 161 were obtained: a 0.6-kb, a 1.8-kb and a 4.8-kb insert fragment.

The insert corresponds to an internal section of the mouse ribosomal RNA gene (rDNA) repeat unit between positions 7551-15670 as set forth in GENBANK accession no. X82564, which is provided as SEQ ID NO. 18. The sequence data obtained for the insert of clone no. 161 is set forth in SEQ ID NOS. 19-25. Specifically, the individual subclones corresponded to the following positions in GENBANK accession no. X82564 (SEQ ID NO:18) and in SEQ ID NOs. 19-25:

Subclone	Start	End	Site	SEQ ID No.
	in X82564			
161k1	7579	7755	*Not*I, *Bam*HI	19
161m5	7756	8494	*Bam*HI	20
161m7	8495	10231	*Bam*HI	21(shows only sequence corresponding to nt. 8495-8950), 22 (shows only sequence corresponding to nt. 9851- 10231)
161m12	10232	15000	*Bam*HI	23 (shows only sequence corresponding to nt. 10232-10600), 24 (shows only sequence corresponding to nt. 14267-15000)
161k2	15001	15676	*Not*I, *Bam*HI	25

The sequence set forth in SEQ ID NOs. 19-25 diverges in some positions from the sequence presented in positions 7551-15670 of GENBANK accession no. X82564. Such divergence may be attributable to random mutations between repeat units of rDNA.

For use herein, the rDNA insert from the clone was prepared by digesting the cosmid with *Not*I and *Bgl*II and was purified as described above. Growth and maintenance of bacterial stocks and purification of plasmids were performed using standard well known methods (see, *e.g.*, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory Press), and plasmids were purified from bacterial cultures using Midi - and Maxi-preps Kits (Qiagen, Mississauga, Ontario).

pDsRed1N1

This vector is available from Clontech (see SEQ ID No. 29) and encodes the red fluorescent protein (DsRed; Genbank accession no. AF272711; SEQ ID Nos. 39 and 40). DsRed, which has a vivid red fluorescence, was isolated from the IndoPacific sea anemone relative *Discosoma* species. The plasmid pDsRed1N1 (Clontech; SEQ ID No. 29) constitutively expresses a human codon-optimized variant of the

fluorescent protein under control of the CMV promoter. Unmodified, this vector expresses high levels of DsRed1 and includes sites for creating N-terminal fusions by cloning proteins of interest into the multiple cloning site (MCS). It is Kan and Neo resistant for selection in bacterial or eukaryotic cells.

Plasmid pMG

Plasmid pMG (InvivoGen, San Diego, California; see SEQ. ID. NO. 27 for the nucleotide sequence of pMG) contains the hygromycin phosphotransferase gene under the control of the immediate-early human cytomegalovirus (hCMV) enhancer/promoter with intron A. Vector pMG also contains two transcriptional units allowing for the coexpression of two heterologous genes from a single vector sequence.

The first transcriptional unit of pMG contains a multiple cloning site for insertion of a gene of interest, the hygromycin phosphotransferase gene (*hph*) and the immediate-early human cytomegalovirus (hCMV) enhancer/promoter with intron A (see, *e.g.*, Chapman *et al.* (1991) *Nuc. Acids Res. 19*:3979-3986) located upstream of *hph* and the multiple cloning site, which drives the expression of *hph* and any gene of interest inserted into the multiple cloning site as a polycistronic mRNA. The first transcriptional unit also contains a modified EMCV internal ribosomal entry site (IRES) upstream of the *hph* gene but downstream of the hCMV promoter and MCS for ribosomal entry in translation of the *hph* gene (see SEQ ID NO. 27, nucleotides 2736-3308). The IRES is modified by insertion of the constitutive *E. coli* promoter (EM7) within an intron (IM7) into the end of the IRES. In mammalian cells, the *E. coli* promoter is treated as an intron and is spliced out of the transcript. A polyadenylation signal from the bovine growth hormone (bGh) gene (see, *e.g.*, Goodwin and Rottman (1992) *J. Biol. Chem. 267*:16330-16334) and a pause site derived from the 3′ flanking region of the human $\alpha 2$

globin gene (see, *e.g.*, Enriquez-Harris *et al.* (1991) *EMBO J.10*:1833-1842) are located at the end of the first transcription unit. Efficient polyadenylation is facilitated by inserting the flanking sequence of the bGh gene 3′ to the standard AAUAAA hexanucleotide sequence.

The second transcriptional unit of pMG contains another multiple cloning site for insertion of a gene of interest and an EF-1α/HTLV hybrid promoter located upstream of this multiple cloning site, which drives the expression of any gene of interest inserted into the multiple cloning site. The hybrid promoter is a modified human elongation factor-1 alpha (EF-1 alpha) gene promoter (see, *e.g.*, Kim *et al.* (1990) *Gene 91*:217-223) that includes the R segment and part of the U5 sequence (R-U5′) of the human T-cell leukemia virus (HTLV) type I long terminal repeat (see, *e.g.*, Takebe *et al.* (1988) *Mol. Cell. Biol 8*:466-472). The Simian Virus 40 (SV40) late polyadenylation signal (see Carswell and Alwine (1989) *Mol. Cell. Biol. 9*:4248-4258) is located downstream of the multiple cloning site. Vector pMG contains a synthetic polyadenylation site for the first and second transcriptional units at the end of the transcriptional unit based on the rabbit β-globin gene and containing the AATAAA hexanucleotide sequence and a GT/T-rich sequence with 22-23 nucleotides between them (see, *e.g.*, Levitt *et al.* (1989)*Genes Dev. 3*:1019-1025). A pause site derived from the C2 complement gene (see, Moreira *et al.* (1995) *EMBO J. 14*:3809-3819) is also located at the 3′ end of the second transcriptional unit.

Vector pMG also contains an ori sequence (ori pMB1) located between the SV40 polyadenylation signal and the synthetic polyadenylation site.

EXAMPLE 2

A. Construction of targeting vector and transfection into LMtk- cells for the generation of platform chromosomes

A targeting vector derived from the vector pWE15 (GeneBank Accession # X65279) was modified by replacing the *Sal*I (Klenow filled)/*Sma*I neomycin resistance containing fragment with the *Pvu*II/*Bam*HI (Klenow filled) puromycin resistance containing fragment (isolated from plasmid pPUR, Clontech Laboratories, Inc. Palo Alto, CA; SEQ ID No. 30) resulting in plasmid pWEPuro. Subsequently a 9 Kb *Not*I fragment from the plasmid pFK161 (SEQ ID NO: 118) containing a portion of the mouse rDNA region was cloned into the *Not*I site of pWEPuro resulting in plasmid pWEPuro9K (Figure 2). The vector pWEPuro9K was digested with *Spe*I to linearize and transfected into LMtk- mouse cells. Puromycin resistant colonies were isolated and subsequently tested for artificial chromosome formation via fluorescent *in situ* hybridization (FISH) (using mouse major and minor DNA repeat sequences, the puromycin gene and telomeres sequences as probes), and fluorescent activated cell sorting (FACS). From this sort, a subclone was isolated containing an artificial chromosome, designated 5B11.12, which carries 4-8 copies of the puromycin resistance gene contained on the pWEPuro9K vector. FISH analysis of the 5B11.12 subclone demonstrated the presence of telomeres and mouse minor on the *ACes*. DOT PCR has been done on the 5B11.12 *ACes* revealing the absence of uncharacterized euchromatic regions on the *ACes*. A recombination site, such as an *att* or *loxP* engineering site or a plurality thereof, was introduced onto this *ACes* thereby providing a platform for site-specific introduction of heterologous nucleic acid.

B. Targeting a single sequence specific recombination site onto platform chromosomes

After the generation of the 5B11.12 platform, a single sequence-specific recombination site is placed onto the platform chromosome via homologous recombination. For this, DNA sequences containing the site-

specific recombination sequence can be flanked with DNA sequences of homology to the platform chromosome. For example, using the platform chromosome made from the pWEPuro9K vector, mouse rDNA sequences or mouse major satellite DNA can be used as homologous sequences to target onto the platform chromosome. A vector is designed to have these homologous sequences flanking the site-specific recombination site and, after the appropriate restriction enzyme digest to generate free ends of homology to the platform chromosome, the DNA is transfected into cells harboring the platform chromosome (Figure 3). Examples of site-specific cassettes that are targeted to the platform chromosome using either mouse rDNA or mouse major repeat DNA include the SV40-attP-hygro cassette and a red fluorescent protein (RFP) gene flanked by loxP sites (Cre/lox, see, *e.g.*, U.S. Patent No. 4,959,317 and description herein). After transfection and integration of the site-specific cassette, homologous recombination events onto the platform chromosome are subcloned and identified by FACS (e.g. screen and single cell subclone via expression of resistance or fluorescent marker) and PCR analysis.

For example, a vector can be constructed containing regions of the mouse rDNA locus flanking a gene cassette containing the SV40 early reporter-bacteriophage lambda attP site-hygromycin selectable marker (see Figure 4 and described below). The use of the bacteriophage lambda attP site for lambda integrase-mediated site-specific recombination is described below. Homologous recombination event of the SV40-attP-hygro cassette onto the platform chromosome was identified using PCR primers that detect the homologous recombination and further confirmed by FISH analysis. After identifying subcloned colonies containing the platform chromosome with a single site-specific recombination site, cells carrying the platform chromosome with a single site-specific

recombination site can now be engineered with site-specific recombinases (e.g. lambda INT, Cre) for integrating a target gene expression vector.

C. Targeting a red fluorescent protein (RFP) gene flanked by loxP sites onto 5B11.12 platform

As another example, while loxP recombination sites could have been introduced onto the *ACes* during *de novo* biosynthesis, it was thought that this might result in multiple segments of the *ACes* containing a high number of loxP sites, potentially leading to instability upon *Cre*-mediated recombination. A gene targeting approach was therefore devised to introduce a more limited number of loxP recombination sites into a locus of the 5B11-12 *ACes* containing introduced and possibly co-amplified endogenous rDNA sequences. Although there are more than 200 copies of rDNA genes in the haploid mouse genome distributed amongst 5-11 chromosomes (depending on strain), rDNA sequences were chosen as the target on the *ACes* since they represent a less frequent target than that of the satellite repeat sequences. Moreover, having observed much stronger pWEPuro9K hybridization to the 5B11-12 *ACes* than to other LMTK$^-$ chromosomes and in light of the observation that the transcribed spacer sequences within the rDNA may be less conserved than the rRNA coding regions, it was contemplated that a targeting vector based on the rDNA gene segment in pWEPuro9K would have a higher probability of targeting to the *ACes* rather than to other LMTK$^-$ chromosomes. Accordingly, a targeting vector, pBSFKLoxDsRedLox, was designed and constructed based on the rDNA sequences contained in pWEPuro9K.

The plasmid pBSFKLoxDsRedLox was generated in 4 steps. First, the *Not*I rDNA insert of pWEPuro9K (Figure 2) was inserted into pBS SK- (Stratagene) giving rise to pBSFK. Second, a loxP polylinker cassette was

generated by PCR amplification of pNEB193 (SEQ ID NO:32; New England Biolabs) using primers complementary to the M13 forward and reverse priming sites at their 3'end and a 34 bp 5' extension comprising a LoxP site. This cassette was reinserted into pNEB193 generating p193LoxMCSLox. Third, the DsRed gene from pDsRed1-N1 (SEQ ID NO:29; Clontech) was then cloned into the polylinker between the loxP sites generating p193LoxDsRedLox. Fourth, a fragment consisting of the DsRed gene flanked by loxP sites was cloned into a unique *NdeI* within the rDNA insert of pBSFK generating pBSFKLoxDsRedLox.

A gel purified 11 Kb *PmlI* /*Eco*RV fragment of pBSFKLoxDsRedLox was used for transfection. To detect targeted integration, PCR primers were designed from rDNA sequences within the 5' *NotI*-*PmlI* fragment of pWEPuro9K that is not present on the targeting fragment (5'primer) and sequence within the LoxDsRedLox cassette (3' primer). If the targeting DNA integrated correctly within the rDNA sequences, PCR amplification using these primers would give rise to a 2.3 Kb band. PCR reactions containing 1-4 μl of genomic DNA were carried out according to the MasterTaq protocol (Eppendorf), using murine rDNA 5' primer (5'-CGGACAATGCGGTTGTGCGT-3'; SEQ ID NO:72) and DsRed 3'primer (5'GGCCCCGTAATGCAGAAGAA-3'; SEQ ID NO:73) and PCR products were analyzed by agarose gel electrophoresis.

$1.5X10^6$ 5B11-12 LMTK$^-$ cells were transfected with 2 μg of the pBSFKLoxDsRedLox targeting DNA described above using Lipofectamine Plus (Invitrogen). For flow sorting, harvested cells were suspended in medium and applied to the Becton Dickinson Vantage SE cell sorter, equipped with 488 nm lasers for excitation and 585/42 bandpass filter for optimum detection of RFP fluorescence. Cells were sorted using dPBS as sheath buffer. Negative control parental 5B11-12 cells and a positive control LMTK$^-$ cell line stably transfected with DsRed were used to

establish the selection gates. The RFP positive gated populations were recovered, diluted in medium supplemented with 1X penicillin-streptomycin (Invitrogen), then plated and cultured as previously described. After 4 rounds of enrichment, the percentage of RFP positive cells reached levels of 50% or higher. DNA from populations was analyzed by PCR for evidence of targeted integration. Ultimately, single cell subclones were established from positive pools and were analyzed by PCR and PCR-positive clones confirmed by FISH as described below. DNA was purified from pools or single cell clones using previously described methods set forth in Lahm et al., Transgenic Res., 1998; 7:131-134, or in some cases using a Wizard Genomic DNA purification kit (Promega). For FISH analysis, a biotinylated DsRed gene probe was generated by PCR using DsRed specific primers and biotin-labeled dUTP (5' RFP primer: 5'-GGTTTAAAGTGCGCTCCTCCAAGAACGTCATC-3', SEQ ID NO:74; and 3' RFP primer: 5'AGATCTAGAGCCGCCGCTACAGGAACAGGTGGTGGCGGCC-3'; SEQ ID NO:75). To maximize the signal intensity of the DsRed probe, Tyramide amplification was carried out according to the manufacturers protocols (NEN).

The process of testing the feasibility of a more general targeting strategy that would not rely on enrichment *via* drug selection of stably transfected clones can be summarized as follows. A red fluorescent protein gene (RFP; encoded by the DsRed gene) was inserted between the loxP sites of the targeting vector to form pBSFKLoxDsRedLox. After transfection with PBSFKLoxDsRedLox, sequential rounds of high speed flow sorting and expansion of sorted cells in culture could then be used to enrich for stable transformants expressing RFP. In the event of targeted integration, PCR screening with primers that amplify from a spacer region within the segment of the 45s pre-rRNA gene in pWEPuro9K to a specific

anchor sequence within the DsRed gene in the targeting cassette would give rise to a diagnostic 2.3 Kb band. However, as rDNA clusters are found on several chromosomes, confirmation of targeting to an *ACes* would require fluorescence in situ hybridization (FISH) analysis. Finally, the flanking of the DsRed gene by loxP sites would allow for its removal and subsequent replacement with other genes of interest.

After transfection of the targeting sequence into 5B11-12 cells, enrichment for targeted clones was carried out using a combination of flow cytometry to detect red-fluorescing cells and PCR screening. Ultimately 17 single cell subclones were identified as potential targeted clones by PCR and of these 16 were found by FISH to contain the DsRed integration event into the *ACes*. These subclones are referred to herein as D11-C4, D11-C12, D11-H3, C9-C9, C9-B9, C9-F4, C9-H8, C9-F2, C9-G8, C9-B6, C9-G3, C9-E12, C9-A11, C11-E3, C11-A9 and C11-H4. PCR analysis of genomic DNA isolated from the D11-C4 subclone gave rise to a 2.3 Kb band, indicative of a targeted integration into an rDNA locus. Further analysis of the subclone by FISH analysis with a DsRed gene probe demonstrated integration of the LoxDsRedLox targeting cassette on the *ACes* co-localizing with one of the regions of rDNA staining seen on the 5B11-12 *ACes*, consistent with a targeted integration into an rDNA locus of the *ACes*, while integrations on other chromosomes were not observed. Since transfected cells were maintained as heterogeneous populations through several cycles of sorting and replating it was not possible to estimate the frequency of targeted events. In most mammalian cell lines the frequency of gene targeting via homologous recombination is roughly 10^{-5}-10^{-7} treated cells. Despite the low frequency of these events in mammalian cells, it is clear that an RFP expression based screening paradigm, coupled with PCR analysis, can effectively detect and enrich for such infrequent events in a large

population. In instances where drug selection is not possible or not desirable, such a system may provide a useful alternative. It was also verified that the modified *ACes* in subclone D11-C4 could be purified by flow cytometry. The results indicate that the flow karyogram of the D11-C4 subclone was unaltered from that of the 5B11-12 cell line. Thus, the D11-C4 *ACes* can be purified in high yield from native chromosomes of the host cell line.

D. Reduction of LoxP on *ACes* to a single site.

The strong hybridization signal detected by FISH on the *ACes* using the DsRed gene probe suggests that several copies of the targeting cassette may be present on the *ACes* in the D11-C4 line. This also suggests that multiple rDNA genes have been correctly targeted.

Accordingly, in certain embodiments where necessary, the number of loxP sites on the *ACes* can be reduced to a single site by *in situ* treatment with *Cre* recombinase, provided that the sites are co-linear. Such a process is described for multiple loxP-flanked integrations on a native mouse chromosome (Garrick et al., Nature Genet., 1998, Jan;18(1):56-59). Reduction to a single loxP site on the D11-C4 *ACes* would result in the loss of the DsRed gene, forming the basis of a useful screen for this event.

For this purpose, a Cre expression plasmid pCX-Cre/GFP III has been generated by first deleting the EcoRI fragment of pCX-eGFP (SEQ ID NO:71) containing the eGFP coding sequence and replacing it with that of a PCR amplified Cre recombinase coding sequence (SEQ ID NO:58), generating pCX-Cre. Next, the AseI/SspI fragment of pD2eGFP-N1 (containing the CMV promoter driving the D2EGFP gene with SV40 polyA signal; Clontech; SEQ ID NO:87) was inserted into the filled HindIII site of pCX-Cre, generating pCX-Cre\GFP III. Control plasmid pCX-CreRev\GFP

III was generated in similar fashion except that the Cre recombinase coding sequence was inserted in the antisense orientation. LMTK⁻ cell line D11-C4 (containing first generation platform *ACes* with multiple loxP-DsRED sites) and 5B11-12 cell line (containing *ACes* with no loxP-DsRED sites) are maintained in culture as described above. D11C4 cells are transfected with 2 μg of plasmid pCX-Cre\GFP III or 2 μg pCX-CreRev\GFP III using Lipofectamine (Invitrogen) as previously described.

Forty-eight to seventy-two hours after transfection, transfected D11-C4 cells are harvested and GFP positive cells are sorted by cell cytometry using a FACSta Vantage cell sorter (Beckton-Dickinson) as follows: All D11-C4 cells transfected with pCX-Cre\GFP III or control plasmid pCX-CreRev\GFP III that exhibit GFP fluorescent higher than the gate level established by untransfected cells are collected and placed in culture a further 7-14 days. After 7-14 days the initial D11-C4 cells are harvested and analyzed by cell cytometry as follows: Untransfected D11-C4 cells are used to establish the gate that defines the RFP positive population, while 5B11-12 cells are used to set the RFP negative gate. The GFP positive population of D11-C4 transfected with pCX-Cre\GFP III should show decreased red fluorescence compared to pCX-CreRev\GFP III transfected or untransfected control D11-C4 cells. The cells exhibiting greatly decreased or no RFP expression are collected and single cell clones subsequently established. These clones will be expanded and analyzed by fluorescence *in-situ* hybridization and Southern blotting to confirm the removal of loxP-DsRed gene copies.

EXAMPLE 3

Construction of targeting vector and transfection into LMtk- cells for the generation of platform chromosomes containing multiple site-specific recombination sites

An example of a selectable marker system for the creation of a chromosome-based platform is shown in Figure 4. This system includes a vector containing the SV40 early promoter immediately followed by (1) a 282 base pair (bp) sequence containing the bacteriophage lambda attP site and (2) the puromycin resistance marker. Initially a *Pvu*II/*Stu*I fragment containing the SV40 early promoter from plasmid pPUR (Clontech Laboratories, Inc., Palo Alto, CA; Seq ID No. 30) was subcloned into the *EcoR*I/CRI site of pNEB193 (a PUC19 derivative obtained from New England Biolabs, Beverly, MA; SEQ ID No. 32) generating the plasmid pSV40193. The only differences between pUC19 and pNEB193 are in the polylinker region. A unique AscI site (GGCGCGCC) is located between the *BamH*I site and the SmaI site, a unique PacI site (TTAATTAA) is located between the *BamH*I site and the *Xba*I site and a unique PmeI site (GTTTAAAC) is located between the *Pst*I site and the *Sal*I site.

The attP site was PCR amplified from lambda genome (GenBank Accession # NC 001416) using the following primers:

attPUP: CCTTGCGCTAATGCTCTGTTACAGG SEQ ID No. 1

attPDWN: CAGAGGCAGGGAGTGGGACAAAATTG SEQ ID No. 2

After amplification and purification of the resulting fragment, the attP site was cloned into the *Sma*I site of pSV40193 and the orientation of the attP site was determined by DNA sequence analysis (plasmid pSV40193attP). The gene encoding puromycin resistance (Puro) was isolated by digesting the plasmid pPUR (Clontech Laboratories, Inc. Palo Alto, CA) with *Age*I/*BamH*I followed by filling in the overhangs with Klenow and subsequently cloned into the *Asc*I site downstream of the attP site of pSV40193attP generating the plasmid pSV40193attPsensePUR (Figure 4; SEQ ID NO:113)).

The plasmid pSV40193attPsensePUR was digested with *Sca*I and co-transfected with the plasmid pFK161 (SEQ ID NO: 118) into mouse LMtk- cells and platform artificial chromosomes were identified and isolated as described above. The process for generating this exemplary platform *ACes* containing multiple site-specific recombination sites is summarized in Figure 5. One platform *ACes* resulting from this experiment is designated B19-18. This platform *ACes* chromosome may subsequently be engineered to contain target gene expression nucleic acids using the lambda integrase mediated site-specific recombination system as described herein in Example 7 and 8.

EXAMPLE 4

Lambda integrase mediated site-specific recombination of a RFP expressing vector onto artificial chromosomes

In this example, a vector expressing the red fluorescent protein (RFP) was produced and recombined into the attP site residing on an artificial chromosome within LMTK- cells. This recombination is depicted in Figure 7.

A. Construction of expression vectors containing wildtype and mutant lambda integrase

Mutations at the glutamic acid at position 174 in the lambda integrase protein relaxes the requirement for the accessory protein IHF during recombination and DNA supercoiling *in vitro* (see, Miller *et al.* (1980) *Cell 20*:721-729; Lange-Gustafson *et al.* (1984) *J. Biol. Chem. 259*:12724-12732). Mutations at this site promote attP, attB intramolecular recombination in mammalian cells (Lorbach *et al.* (2000) *J. Mol. Biol 296*:1175-1181).

To construct nucleic acid encoding the mutant, lambda integrase was PCR amplified from bacteriophage lambda DNA (cI857 *ind Sam* 7; New England Biolabs) using the following primers:

Lamint1 (SEQ ID No. 3)

TTCGAATTCATGGGAAGAAGGCGAAGTCATGAGCG)
Lamint2 (SEQ ID No. 4)
(TTCGAATTCTTATTTGATTTCAATTTTGTCCCAC).
The resulting PCR product was digested with *Eco*R I and cloned into the *Eco*R I site of pUC19. Lambda integrase was mutated at amino acid position 174 using QuikChange Site-Directed Mutagenesis Kit (Stratagene) and the following oligos (generating a glutamic acid to arginine change at position 174):
LambdaINTE174R
(SEQ ID No. 6)
(CGCGCAGCAAAATCTAGAGTAAGGAGATCAAGACTTACGGCTGACG),
LamintR174rev (SEQ ID No. 7)
(CGTCAGCCGTAAGTCTTGATCTCCTTACTCTAGATTTTGCTGCGCG).
The resulting site directed mutant was confirmed by sequence analysis. The wildtype and mutant lambda genes were cloned into the *Eco*R I site of pCX creating pCX-LamInt (SEQ ID NO: 127) and pCXLamIntR (Figure 8; SEQ ID NO: 112).

The plasmid pCX (SEQ ID No. 70) was derived from plasmid pCXeGFP (SEQ ID No. 71). Excision of the EcoRI fragment containing the eGFP marker generated pCX. To generate plasmid pCXLamINTR (SEQ ID NO: 112) an EcoRI fragment containing the lambda integrase E174R (SEQ ID No. 37) mutation was cloned into the *Eco*RI site of pCX, and to generate plasmid pCX-LamINT, an *Eco*RI fragment containing the wild-type lambda integrase was cloned into the *Eco*RI site of pCX.

B. Construction of integration vector containing attB and DsRed

The plasmid pDsRedN1 (Clontech Laboratories, Palo Alto, CA; SEQ ID No. 29) was digested with *Hpa* I and ligated to the following annealed oligos:
attB1 (SEQ ID No. 8)

(TGAAGCCTGCTTTTTTATACTAACTTGAGCGAA)

attB2 (SEQ ID No. 9)

(TTCGCTCAAGTTAGTATAAAAAAGCAGGCTTCA)

The resulting vector (pDsRedN1-attB) was confirmed by PCR and sequence analysis.

C. **Transfection into LMtk- cells**

LM(tk-) cells containing the Prototype A *ACes* (L1-18; Chromos Molecular Systems Inc., Burnaby, BC Canada) were co-transfected with pDsRedN1 or pDsRedN1-attB and either pCXLamInt (SEQ ID NO: 127) or pCXLamIntR (SEQ ID NO: 112) using Lipofectamine Plus Reagent (LifeTechnologies, Gaithersburg, MD). The transfected cells were grown in DMEM (LifeTechnologies, Gaithersburg, MD) with 10% FBS (CanSera) and G418 (CalBiochem) at a concentration of 1 mg/ml.

D. **Enrichment by cell sorting**

The transfected cells were sorted using a FACs Vantage SE cell sorter (Becton Dickenson) to enrich for cells expressing DsRed. The cells were excited with a 488 nm Argon laser at 200 watts and cells fluorescing in the 585/42 detection channel were collected. The sorted cells were returned to growth medium for recovery and expansion. After three successive enrichments for cells expressing DsRed, single cell sorting into 96 well plates was performed using the same parameters. Duplicate plates of the single cell clones were made for PCR analysis.

E. **PCR analysis of single cell clones**

Pools of cells from each row and column of the 96 well plate were used for DNA isolation. DNA was prepared using a Wizard Genomic DNA purification kit (Promega Inc, Madison, WI). Nested PCR analysis on the DNA pools was performed to confirm the site-specific recombination event using the following primer sets:

attPdwn2 (SEQ ID No. 10)
(TCTTCTCGGGCATAAGTCGGACACC)

CMVen (SEQ ID No. 11)
(CTCACGGGGATTTCCAAGTCTCCAC)

followed by:

attPdwn (SEQ ID No. 12)
(CAGAGGCAGGGAGTGGACAAAATTG)

CMVen2 (SEQ ID No. 13)
(CAACTCCGCCCCATTGACGCAAATG).

The resulting PCR reactions were analyzed by gel electrophoresis and the potential individual clones containing the site-specific recombination event were identified by combining the PCR results of all of the pooled rows and columns for each 96 well plate. The individual clones were then further analyzed by PCR using the following primers that flank the recombination junction. L1for and F1rev flank the attR junction whereas REDfor and L2rev flank the attL junction (see Figure 7):

L1for (SEQ ID No. 14)
AGTATCGCCGAACGATTAGCTCTTCA

F1rev (SEQ ID No. 15)
GCCGATTTCGGCCTATTGGTTAAA

REDfor (SEQ ID No. 16)
CCGCCGACATCCCCGACTACAAGAA

L2rev (SEQ ID No. 17)
TTCCTTCGAAGGGGATCCGCCTACC.

F. Sequence analysis of recombination junctions

PCR products spanning the recombination junction were Topo-cloned into pcDNA3.1D/V5His (Invitrogen Inc., San Diego, CA) and then sequenced by cycle-sequencing. The clones were confirmed to have the correct *att*R and *att*L junctions by cycle sequencing.

G. Fluorescent In Situ Hybridization (FISH)

The cell lines containing the correct recombination junction sequence were further analyzed by fluorescent *in situ* hybridization (FISH)

by probing with the DsRed coding region labeled with biotin and visualizing with the Tyramide Signal Amplification system (TSA; NEN Life Science Products). The results indicate that the RFP sequence is present on the *ACes*.

H. *Southern analysis*

Genomic DNA was harvested from the cell lines containing an *ACes* with the correct recombinant event and digested with *Eco*R I. The digested DNAs were separated on a 0.7% agarose gel, transferred and fixed to a nylon membrane and probed with RFP coding sequences. The result showed that there is an integrated copy of RFP coding sequence in each clone.

EXAMPLE 5

Delivery of a second gene encoding GFP onto the RFP platform *ACes*

A. Construction of integration vector containing attB and GFP (pD2eGFPIresPuroattB).

The plasmid pIRESpuro2 (Clontech, Palo Alto, CA; SEQ ID NO: 88) was digested with *Eco*RI and *Not*I then ligated to the D2eGFP *Eco*RI-*Not*I fragment from pD2eGFP-N1 (Clontech, Palo Alto, CA) to create pD2eGFPIresPuro2. Subsequently, oligos encoding the attB site were annealed and ligated into the *Nru*I site of pD2eGFPIresPuro2 to create pD2eGFPIresPuroattB. The orientation of attB in the *Nru*I site was determined by PCR.

B. *Transfection of LMtk- cells*

The LMtk- cells containing the RFP platform *ACes* produced in Example 4, which has multiple attP sites, were co-transfected with pCXLamIntR and pD2eGFPIresPuroattB using LipofectAMINE PLUS reagent. Five μg of each vector was placed into a tube containing 750 μl of DMEM (Dulbecco's modified Eagles Medium). Twenty μl of the Plus reagent was added to the DNA and incubated at room temperature for 15

minutes. A mixture of 30 μl of lipofectamine and 750 μl DMEM was added to the DNA mixture and incubated an additional 15 minutes at room temperature. The DNA mixture was then added dropwise to approximately 3 million cells attached to a 10cm dish in 5 mls of DMEM. The cells were incubated 4 hours (37°C, 5% CO_2) with the DNA-lipid mixture, after which DMEM with 20% fetal bovine serum was added to the dishes to bring the culture medium to 10% fetal bovine serum. The dishes were incubated at 37°C with 5% CO_2.

Plasmid pD2eGFPIresPuroattB has a puromycin gene transcriptionally linked to the GFP gene *via* an IRES element. Two days after the transfection the cells were placed in medium containing puromycin at 4μg/ml to select for cells containing the pD2eGFPIresPuroattB plasmid integrated into the genome. Twenty-three clones were isolated after 17 days of selection with puromycin. These clones were expanded and then analyzed for the presence of the GFP gene on the *ACes* by 2-color (RFP/biotin & GFP/digoxigenin) TSA-FISH (NEN) according to the manufacturers protocol. Sixteen of the 23 clones produced a positive FISH signal on the *ACes* with a GFP probe.

EXAMPLE 6

Delivery Of *ACes* Into human Mesenchymal Stem Cells (hMSC)

A. Transfection

Transfection conditions for the most efficient delivery of the *ACes* into hMSCs (Cambrex BioWhittaker Product Code PT-2501, lot# F0658, East Rutherford, New Jersey) were assayed using LipofectAMINE PLUS and Superfect. One million prototype B *ACes*, which is a murine derived 60Mb *ACes* having primarily murine pericentric heterochromatin, and carrying a "payload" containing a hygromycin B selectable marker gene and a *lac*Z reporter gene (see , Telenius et al., 1999, Chrom. Res., 7:3-7; and Kereso et al., 1996, Chrom. Res., 4:226-239; each of which is

incorporated herein by reference in its entirety), were combined with 1-12 μl of the transfection agent. In the case of LipofectAMINE PLUS, the PLUS reagent was combined with the *ACes* for 15 minutes followed by LipofectAMINE for a further 15 minutes. Superfect was complexed for 10 minutes at a ratio of 2μl Superfect per 1 million *ACes*. The *ACes*/transfection agent complex was then applied to 0.5 million recipient cells and the transfection was allowed to proceed according to the manufacturer's protocol. Percent transfected cells was determined on a FACS Vantage flow cytometer with argon laser tuned to 488 nm at 200mW and FITC fluorescence collected through a standard FITC 530/30 nm band pass filter. After 24 hours, IdUrd labeled *ACes* were delivered to human MSCs in the range of 30-50%, varying with transfection agent and dose. *ACes* delivery curves were generated from data collected in experiments that varyied the dose of the transfection reagents. Dose response curves of Superfect and LipofectAMINE PLUS, showing delivery of *ACes* into recipient hMSCs cells, were prepared, measured by transfer of IdUrd labeled *ACes* and detected by flow cytometry. Superfect shows maximum delivery in the range of 30-50% at doses greater than 2 μl per million *ACes*. LipofectAMINE PLUS has a 42-48% delivery peak around 5-8 μl per million *ACes*. These dose curves were then correlated with toxicity data to determine the transfection conditions that will allow for highest potential transfection efficiency. Toxicity was determined by a modified plating efficiency assay (de Jong et al., 2001, Chrom. Research, 9:475-485). The population's normalized plating efficiency (at maximum % delivery doses) was in the range of 0.2 - 0.4 for Superfect and 0.5 - 0.6 with LipofectAMINE PLUS.

Due to the transfected population consisting of mixed cell types, flow cytometry allowed for the assessment of *ACes* delivery into each sub-population and the purification of the target population. Flow profiles

showing forward scatter (cell size) and side scatter (internal cell granularity) revealed three distinct hMSC populations that were gated into three regions: R3 (small cell region), R4 (medium cell region), R5 (large cell region). Transfection conditions were further optimized by re-analyzing delivery curves and assessing the differences in delivery to each sub-population. Dose response curves of Superfect and LipofectAMINE were prepared showing % delivery to each sub-population represented by the gating on basis of cell size and granularity properties of the mixed population. Three distinct hMSC populations were gated and % delivery dose curves generated. Using Superfect and LipofectAMINE PLUS the overall % delivery increased with cell size (80-90% delivery in large cells). LipofectAMINE PLUS at high doses (8-12 μl per 1 million *ACes*) shows an increase in the overall proportion of chromosome transfer to the small population (10-20%). This suggests an advantage to using this transfection agent if the small-undifferentiated cell population is the desired target host cell.

B. Expression from Genes on *ACes* IN hMSCs

Following the delivery screening process conducted in section (A) above, the most promising results were subjected to further analyses to monitor expression and verify the presence of structurally intact *ACes*. The transfection conditions employed for these experiments were exactly the same as those that had been used during the screening process. Short-term expression was monitored by transfecting hMSCs with *ACes* containing a RFP gene (red fluorescent protein) set forth in Example 2C as "D11C4". The unselected population was harvested at 72-96 hours post transfection and % positive fluorescent cells measured by flow cytometry. RFP expression was in the range of 1-20%.

Long term-gene expression was assayed by selecting for hygromycin B resistant cells over a period of 7-10 days. Cytogenetic

analysis was done to detect presence of intact *ACes* by Fluorescent *In Situ* hybridization (FISH), where metaphase chromosomes were hybridized to a mouse major satellite-DNA probe (targeting murine pericentric heterochromatin) and a lambda probe (hybridizing to the *lac*Z gene). The human mesenchymal transfected culture could not undergo standard sub-cloning as diffuse colonies form with limited doublings available for expansion. Cytogenetic analysis was performed on the entire population, sampling over a period of 3-10 days post-transfection. The hygromycin resistant population was then blocked in mitosis with colchicine and analyzed for presence of intact *ACes* by FISH. Preliminary FISH results show approximately 2-8% of the hMSC-transfected population had an intact *ACes*. This compared to rat skeletal muscle myoblast clones, which were in the range of 60-95%. To increase the % of intact *ACes* in the hMSC-transfected population an enrichment step can be utilized as described in Example 2C.

C. Differentiation of The hMSCs

In initial experiments where transfected hMSCs cells have been induced to differentiate into adipose or osteocytes, the results indicate that the transfected cells appear to be differentiating at a rate comparable to the untransfected controls and the cultures are lineage specific as tested by microscopic examination, FISH, Oil Red O staining (adipocyte assay), and calcium secretion (osteocyte assay).

Accordingly, these results indicate that the artificial chromosomes (*ACes*) provided herein can be successfully transferred into hMSC target cells. Targeting MSCs (such as hMSCs) permits gene transfer into cells in an undifferentiated state where the cells are easier to expand and purify. The genetically modified cells can then be differentiated *in vitro* or injected into a site *in vivo* where the microenvironment will induce transformation into specific cell lineages.

EXAMPLE 7

Delivery of a Promoterless Marker Gene to a Platform *ACes*

Platform *ACes* containing pSV40attPsensePURO (Figure 4) were constructed as set forth in Examples 3 and 4.

A. Construction of Targeting Vectors.

The base vector p18attBZeo (3166bp; SEQ ID NO: 114) was constructed by ligating the 1067bp *Hin*dIII-*Ssp*I fragment containing attBZeo, obtained from pLITattBZeo (SEQ ID NO:91), into pUC18 (SEQ ID NO: 122) digested with *Hin*dIII and *Ssp*I.

1. p18attBZEO-eGFP (6119bp; SEQ ID NO: 126) was constructed by inserting the 2977bp *Spe*I-*Hin*dIII fragment from pCXeGFP (SEQ ID NO:71; Okabe, *et al.* (1997) *FEBS Lett* 407:313-319) containing the eGFP gene into p18attBZeo (SEQ ID NO: 114) digested with *Hin*dIII and *Xba*I.

2. p18attBZEO-5'6XHS4eGFP (Figure 10; 7631bp; SEQ ID NO: 116) was constructed by ligating the 4465bp *Hin*dIII fragment from pCXeGFPattB(6XHS4)2 (SEQ ID NO: 123) which contains the eGFP gene, under the regulation of the chicken beta actin promoter, 6 copies of the HS4 core element located 5' of the chicken beta actin promoter and the polyadenylation signal into the *Hin*dIII site of p18attBZeo (SEQ ID NO: 114).

3. p18attBZEO-3'6XHS4eGFP (Figure 11; 7600bp; SEQ ID NO: 115) was created by removing the 5'6XHS4 element from p18attBZeo-(6XHS4)2eGFP (SEQ ID NO: 110). p18attBZeo-(6XHS4)2eGFP was digested with *Eco*RV and *Spe*I, treated with Klenow and religated to form p18attBZeo3'6XHS4eGFP (SEQ ID NO: 115).

4. p18attBZEO-(6XHS4)2eGFP (Figure 12; 9080bp; SEQ ID NO: 110) was created in two steps. First, the *Eco*RI-*Spe*I fragment from pCXeGFPattB(6XHS4)2 (SEQ ID NO: 123) which contains 6 copies of the HS4 core element was ligated into p18attBZeo (SEQ ID NO: 114)

digested with *Eco*RI and *Xba*I to create p18attBZeo6XHS4 (4615bp; SEQ ID NO: 117). Next, p18attBZeo6XHS4 was digested with *Hin*dIII and ligated to the 4465bp *Hin*dIII fragment from pCXeGFPattB(6XHS4)2 which contains the eGFP gene, under the regulation of the chicken beta actin promoter, 6 copies of the HS4 core element located 5' of the chicken beta actin promoter and the polyadenylation signal.

Table 2

Targeting plasmid	No. zeocin resistant clones	No. clones with expected PCR product size	No. clones with correct sequence at recombination junction
p18attBZEO-eGFP	12	12	NT*
p18attBZEO-5'6XHS4eGFP	11	11	NT
p18attBZEO-3'6XHS4eGFP	11	11	NT
p18attBZEO-(6XHS4)2eGFP	9	9	4/4

*NT = not tested

B. Transfection and Selection with Drug.

The mouse cell line containing the 2nd generation platform ACE, B19-38 (constructed as set forth in Example 3), was plated onto four 10cm dishes at approximately 5 million cells per dish. The cells were incubated overnight in DMEM with 10% fetal calf serum at 37°C and 5% CO_2. The following day the cells were transfected with 5μg of each of the 4 vectors listed in Example 7.A. above and 5μg of pCXLamIntR (SEQ ID NO: 112), for a total of 10μg per 10cm dish. Lipofectamine Plus reagent was used to transfect the cells according to the manufacturers protocol. Two days post-transfection zeocin was added to the medium at 500ug/ml. The cells were maintained in selective medium until colonies formed. The colonies were then ring-cloned (see, e.g., McFarland, 2000, Methods Cell Sci, Mar;22(1):63-66).

C. Analysis of Clones (PCR, SEQUENCING).

Genomic DNA was isolated from each of the candidate clones with the Wizard kit (Promega) and following the manufacturers protocol. The following primer set was used to analyze the genomic DNA isolated from the zeocin resistant clones: 5PacSV40 - CTGTTAATTAACTGTGGAATGTGTG TCAGTTAGGGTG (SEQ ID NO:76); Antisense Zeo - TGAACAGGGTCACGTCGTCC (SEQ ID NO:77). PCR amplification with the above primers and genomic DNA from the site-specific integration of any of the 4 zeocin vectors would result in a 673bp PCR product.

As set forth in Table 2, of the 4 zeocin resistant candidate clones thusfar analyzed by PCR, all 4 exhibit the correct sequence for a site-specific integration event.

EXAMPLE 8

Integration of a PCR product by site-specific recombination.

In this example a gene is integrated onto the platform *ACes* by site-specific recombination without cloning said gene into a vector.

A. PCR PRIMER DESIGN.

PCR primers are designed to contain an attB site at the 5' end of one of the primers in the primer set. The remaining primers, which could be one or more than one primer, do not contain an attB site, but are complementary to sequences flanking the gene or genes of interest and any associated regulatory sequences. In first example, 2 primers (one containing an attB site) are used to amplify a selective gene such as puromycin.

In a second example as shown in Figure 13, the primer set includes primers 1 & 2 that amplify the GFP gene without amplification of an upstream promoter. Primer 1 contains the attB site at the 5' end of the oligo. Primers 3 & 4 are designed to amplify the IRES-blasticidin DNA sequences from the vector pIRESblasticidin. The 5'end of primer 3

contains sequences complementary to the 5′ end of primer 2 such that annealing can occur between 5′ ends of the two primers.

B. PCR REACTION AND SUBSEQUENT LIGATION TO CREATE CIRCULAR MOLECULES FROM THE PCR PRODUCT

In the first example set forth above in Section A, the two PCR primers are combined with a puromycin DNA template such as pPUR (Clontech), a heat stable DNA polymerase and appropriate conditions for DNA amplification. The resulting PCR product (attB-Puromycin) is then then purified and self-ligated to form a circular molecule.

In the second example set forth above in Section A, amplification of the GFP gene and IRES-blasticidin sequences is accomplished by combining primers 1 & 2 with DNA template pD2eGFP and primers 3 & 4 with template pIRESblasticidin under appropriate conditions to amplify the desired template. After initial amplification of the two products (attB-GFP & IRES-blasticidin) in separate reactions, a second round of amplification using both of the PCR products from the first round of amplification together with primers 1 and 4 amplifies the fusion product attB-GFP-IRES-blasticidin (Figure 13). This technique of using complementary sequences in primer design to create a fusion product is employed in *Saccharomyces cerevisiae* for allele replacement (Erdeniz *et al* (1997) ***Gen Res*** 7:1174-1183). The amplified product is then purified from the PCR reaction mixture by standard methods and ligated to form a circular molecule.

C. INTRODUCTION OF PCR PRODUCT ONTO THE *ACes* USING A RECOMBINASE

The circular PCR product is then be introduced to the platform *ACes* using the bacteriophage lambda integrase E174R. The introduction can be performed *in vivo* by transfecting the pCXLamIntR (SEQ ID NO: 112) vector encoding the lambda integrase mutant E174R together with the circularized PCR product into a cell line containing the platform ACE.

D. SELECTION FOR MARKER GENE

The marker gene (in this case either puromycin, blasticidin or GFP) is used to enrich the population for cells containing the proper integration event. A proper integration event in the second example (Figure 14) juxtaposes a promoter residing on the platform *ACes* 5' to the attB-GFP-IRES-Blasticidin PCR product, allowing for transcription of both GFP and blasticidin. If enrichment is done by drug selection, blasticidin is added to the medium on the transfected cells 24-48 hours post-transfection. Selection is maintained until colonies are formed on the plates. If enrichment is done by cell sorting, cells are sorted 2-4 days post-transfection to enrich for cells expressing the fluorescent marker (GFP in this case).

E. ANALYSIS OF CLONES

Clonal isolates are analyzed by PCR, FISH and sequence analysis to confirm proper integration events.

EXAMPLE 9

Construction of a human platform *ACes* "ACE 0.1"

A. CONSTRUCTION OF THE TARGETING VECTOR pPACrDNA

Genome Systems (IncyteGenomics) was supplied with the primers 5'HETS (GGGCCGAAACGATCTCAACCTATT; SEQ ID NO:78), and 3'HETS (CGCAGCGGCCCTCCTACTC; SEQ ID NO:79), which were used to amplify a 538bp PCR product homologous to nt 9680-10218 of the human rDNA sequences (GenBank Accession No. U13369) and used as a probe to screen a human genomic P1AC (P1 Artificial Chromosome) library constructed in the vector pCYPAC2 (Ioannou *et al.* (1994) *Nat. Genet. 6(1):* 84-89). Genome Systems clone #18720 was isolated in this screen and contains three repeats of human rDNA as assessed by restriction analysis. GS clone #18720, was digested with PmeI, a restriction enzyme unique to a single repeat of the human rDNA (45Kbp),

and then religated to form pPACrDNA (Figure 15). The insert in pPACrDNA was analyzed by restriction digests and sequence analysis of the 5′ and 3′ termini. The pPACrDNA, rDNA sequences are homologous to Genbank Accession #U13369, containing an insert of about 45 kB comprising a single repeat beginning from the end of one repeat at ~33980 (relative to the Genbank sequence) through the beginning of the next repeat up to approximately 35120 (the repeat offset from that listed in the GenBank file). Thus, the rDNA sequence is just over 1 copy of the repeat extending from 33980 (+/-10bp) to the end of the first repeat (43Kbp) and continuing into the second repeat to bp 35120 (+/-10bp).

B. TRANSFECTION AND *ACes* FORMATION.

Five hundred thousand MSU1.1 cells (Morgan et al., 1991, Exp. Cell Res., Nov;197(1):125-136; provided by Dr. Justin McCormick at Michigan State University) were plated per 6cm plate (3 plates total) and allowed to grow overnight. The cells were 70-80% confluent the following day. One plate was transfected with 15μg pPACrDNA (linearized with *Pme* I) and 2μg pSV40attPsensePuro (linearized with *Sca* I; see Example 3). The remaining plates were controls and were transfected with either 20μg pBS (Stratagene) or 20μg pSV40attBsensePuro (linearized with *Sca* I). All three plates were transfected using a $CaPO_4$ protocol.

C. SELECTION OF PUROMYCIN RESISTANT COLONIES

One day post-transfection the cells were "glycerol shocked" by the addition of PBS medium containing 10% glycerol for 30 seconds. Subsequently, the glycerol was removed and replaced with fresh DMEM. Four days post-transfection selective medium was added. Selective medium contains 1ug/ml puromycin. The transfection plates were maintained at 37°C with 5% CO_2 in selective medium for 2 weeks at which point colonies could be seen on the plate transfected with

pPACrDNA and pSV40attPsensePuro. The colonies were ring-cloned from the plate on day 17 post-selection and expanded in selective medium for analysis. Only two colonies (M2-2d & M2-2b) were able to proliferate in the selective medium after cloning. No colonies were seen on the control plates after 37 days in selective medium.

D. ANALYSIS OF CLONES

FISH analysis was performed on the candidate clones to detect *ACes* formation. Metaphase spreads from the candidate clones were probed in multiple probe combinations. In one experiment, the probes used were biotin-labeled human alphoid DNA (pPACrDNA) and digoxigenin-labeled mouse major DNA (pFK161) as a negative control. Candidate M2-2d was single cell subcloned by flow sorting and the candidate subclones were reanalyzed by FISH. Subclone 1B1 of M2-2d was determined to be a platform *ACes* and is also designated human Platform ACE 0.1.

EXAMPLE 10

Site-specific integration of a marker gene onto a human platform ACE 0.1

The promoterless delivery method was used to deliver a promoterless blasticidin marker gene onto the human platform *ACes* with excellent results. The human *ACes* platform with a promoterless blasticidin marker gene resulted in 21 of 38 blasticidin resistant clones displaying a PCR product of the expected size from the population co-transfected with pLIT38attBBSRpolyA10 and pCXLamIntR (Figure 8; SEQ ID NOs. 111 and 112). Whereas, the population transfected with pBlueScript resulted in 0 blasticidin resistant colonies.

A. CONSTRUCTION OF pLIT38attB-BSRpolyA10 & pLIT38attB-BSRpolyA2.

The vector pLITMUS 38 (New England Biolabs; U.S. Patent No. 5,691,140; SEQ ID NO: 119) was digested with *Eco*RV and ligated to

two annealed oligomers, which form an attB site (attB1 5'-TGAAGCCTGCTTTTTTATACTAACTTGAGCGAA-3' (SEQ ID NO:8); attB2 5'- TTCGCTCAAGTTAGTATAAAAAAGCAGGCTTCA-3'; SEQ ID NO:9). This ligation reaction resulted in the vector pLIT38attB (SEQ ID NO: 120). The blasticidin resistance gene and SV40 polyA site was PCR amplified with primers: 5BSD (ACCATGAAAACATTTAACATTTCTCAACA; SEQ ID NO:80) and SV40polyA (TTTATTTGTGAAATTTGTGATGCTATTGC; SEQ ID NO:81) using pPAC4 (Frengen, E., *et al.* (2000) Genomics 68 (2), 118-126; GenBank Accession No. U75992) as template. The blasticidin-SV40polyA PCR product was then ligated into pLIT38attB at the *Bam*HI site, which was Klenow treated following digestion with *Bam*HI. pLIT38attB-BSDpolyA10 (SEQ ID NO: 111) and pLIT38attB-BSDpolyA2 (SEQ ID NO: 121) are the two resulting orientations of the PCR product ligated into the vector.

B. TRANSFECTION OF MSU1.1 CELLS CONTAINING HUMAN PLATFORM ACE 0.1.

MSU1.1 cells containing human platform ACE 0.1 (see Example 9) was expanded and plated to five 10cm dishes with 1.3×10^6 cells per dish. The cells were incubated overnight in DMEM with 10% fetal bovine serum, at 37°C and 5% CO_2. The following day the cells were transfected with 5μg of each plasmid as set forth in Table 3, for a total of 10μg of DNA per plate of cells transfected (see Table 3) using ExGen 500 *in vitro* transfection reagent (MBI fermentas, cat. no. R0511). The transfection was performed according to the manufacturers protocol. Cells were incubated at 37°C with 5% CO_2 in DMEM with 10% fetal bovine serum following the transfection.

Table 3

Plate #	Plasmid 1	Plasmid 2	No. Bsd^R Colonies
1	pBS	None	0
2	pCXLamInt	pLIT38attB-BSRpolyA10	16
3	pCXLamIntR	pLIT38attB-BSRpolyA10	40
4	pCXLamInt	pLIT38attB-BSRpolyA2	28
5	pCXLamIntR	pLIT38attB-BSRpolyA2	36

C. SELECTION OF BLASTICIDIN RESISTANT CLONES.

Three days following the transfection the cells were split from a 10 cm dish to two 15cm dishes. The cells were maintained in DMEM with 10% fetal bovine serum for 4 days in the 15 cm dishes. Seven days post-transfection blasticidin was introduced into the medium. Stably transfected cells were selected with 1μg/ml blasticidin. The number of colonies formed on each plate is listed in Table 3. These colonies were ring-cloned and expanded for PCR analysis. Upon expansion in blasticidin containing medium some clones failed to live and therefore do not have corresponding PCR data.

D. PCR ANALYSIS

Thirty-eight of the 40 clones from plate 3 grew after ring-cloning. Genomic DNA was isolated from these clones with the Promega Wizard Genomic cDNA purification kit, digested with *Eco*RI and used as template in a PCR reaction with the following primers: 3BSP – TTAATTTCGGG TATATTTGAGTGGA (SEQ ID NO:82); 5PacSV40 – CTGTTAATTAACTGTGGAA TGTGTGTCAGTTAGGGTG (SEQ ID NO:76). The PCR conditions were as follows. 100ng of genomic DNA was

amplified with 0.5ul Herculase polymerase (Stratagene) in a 50ul reaction that contained 12.5pmole of each primer, 2.5mMof each dNTP, and 1X Herculase buffer (Stratagene). The reactions were placed in a PerkinElmer thermocycler programmed as follows: Initial denaturation at 95°C for 10 minutes; 35 cycles of 94°C for 1minute, 53°C for 1 minute, 72°C for 1 minute, and 72°C for 1 minute; Final extension for 10 minutes at 72°C; and 4°C hold. If pLIT38attB-BSRpolyA10 integrates onto the human platform ACE 0.1 correctly, PCR amplification with the above primers should yield an 804bp product. Twenty-one of the 38 clones from plate 3 produced a PCR product of the expected 804bp size.

EXAMPLE 11

Delivery of a Vector comprising a Promoterless Marker Gene and a gene encoding a therapeutic product to a Platform *ACes*

Platform *ACes* containing pSV40attPsensePURO (Figure 4) were constructed as set forth in Examples 3 and 4.

A. CONSTRUCTION OF DELIVERY VECTORS

1. Erythropoietin cDNA vector, p18EPOcDNA.

The erythropoietin cDNA was PCR amplified from a human cDNA library (E. Perkins *et al.*, 1999, *Proc. Natl. Acad. Sci. USA 96(5)*: 2204-2209) using the following primers: EPO5XBA - TATCTAGAATGGGGGTGC ACGAATGTCCTGCC (SEQ ID NO: 83); EPO3BSI - TACGTACGTCATC TGTCCCCTGTCCTGCAGGC (SEQ ID NO: 84). The cDNA was amplified through two successive rounds of PCR using the following conditions: heat denaturation at 95°C for 3 minutes; 35 cycles of a 30 second denaturation (95°C), 30 seconds of annealing (60°C), and 1 minute extension (72°C); the last cycle is followed by a 7 minute extension at 72°C. BIO-X-ACT (BIOLINE) was used to amplify the erythropoietin cDNA from 2.5ng of the human cDNA library in the first round of amplification. Five μl of the first amplification product was used

as template for the second round of amplification. Two PCR products were produced from the second amplification with Taq polymerase (Eppendorf), each product was cloned into pCR2.1-Topo (Invitrogen) and sequenced. The larger PCR product contained the expected cDNA sequence for erythropoietin. The erythropoietin cDNA was moved from pTopoEPO into p18attBZeo(6XHS4)2eGFP (SEQ ID NO: 110). pTopoEPO was digested with BsiWI and XbaI to release a 588 bp EPO cDNA. BsrGI and BsiWI create compatable ends. The eGFP gene was removed from p18attBZeo(6XHS4)2eGFP by digestion with BsiWI and XbaI, the 8.3 Kbp vector backbone was gel purified and ligated to the 588 bp EPO cDNA to create p18EPOcDNA (SEQ ID NO: 124).

2. Genomic erythropoietin vector, p18genEPO.

The erythropoietin genomic clone was PCR amplified from a human genomic library (Clontech) using the following primers: GENEPO3BSI - CGTACGTCATCTGTCCCCT GTCCTGCA (SEQ ID NO: 85); GENEPO 5XBA -TCTAGAATGGGGGT GCACGGTGAGTACT (SEQ ID NO: 86). The reaction conditions for the amplification were as follows: heat denaturation for 3 minutes (95°C); 30 cycles of a 30 second denaturation (95°C), 30 seconds annealing (from 65°C decreasing 0.5°C per cycle to 50°C), and 3 minutes extension (72°C); 15 cycles of a 30 second denaturation (95°C), 30 seconds annealing (50°C), and 3 minute extension (72°C); the last cycle is followed by a 7 minute extension at 72°C. The erythropoietin genomic PCR product (2147 bp) was gel purified and cloned into pCR2.1Topo to create pTopogenEPO. Sequence analysis revealed 2bp substitutions and insertions in the intronic sequences of the genomic clone of erythropoietin. A partial digest with XbaI and complete digest with BsiWI excised the erythropoietin genomic insert from pTopogenEPO. The resulting 2158 bp genomic erythropoietin fragment was ligated into the 8.3 Kbp fragment resulting from the

digestion of p18attBZeo(6XHS4)2eGFP (SEQ ID NO: 110) with XbaI and BsrGI to create p18genEPO (SEQ ID NO: 125).

B. TRANSFECTION AND SELECTION WITH DRUG

The erythropoietin genomic and cDNA genes were each moved onto the platform *ACes* B19-38 (constructed as set forth in Example 3) by co-transfecting with pCXLamIntR. Control transfections were also performed using pCXLamInt (SEQ ID NO: 127) together with either p18EPOcDNA (SEQ ID NO: 124) or p18genEPO (SEQ ID NO: 125). Lipofectamine Plus was used to transfect the DNA's into B19-38 cells according to the manufacturer's protocol. The cells were placed in selective medium (DMEM with 10% FBS and Zeocin @ 500ug/ml) 48 hours post-transfection and maintained in selective medium for 13 days. Clones were isolated 15 days post-transfection.

C. ANALYSIS OF CLONES (ELISA, PCR)

1. ELISA Assays

Thirty clones were tested for erythropoietin production by an ELISA assay using a monoclonal anti-human erythropoietin antibody (R&D Systems, Catalogue # MAB287), a polyclonal anti-human erythropoietin antibody (R & D Systems, Catalogue # AB-286-NA) and alkaline phosphotase conjugated goat-anti-rabbit IgG (heavy and light chains) (Jackson ImmunoResearch Laboratories, Inc., Catalogue # 111-055-144). The negative control was a Zeocin resistant clone isolated from B19-38 cells transfected with p18attBZeo(6XHS4) (SEQ ID NO: 117; no insert control vector) and pCXLamIntR (SEQ ID NO: 112). The preliminary ELISA assay was executed as follows: 1) Nunc-Immuno Plates (MaxiSorb 96-well, Catalogue # 439454) were coated with 75ul of a 1/200 dilution (in Phosphate buffered Saline, pH 7.4 (PBS), Sigma Catalogue # P-3813) of monoclonal anti-human erythropoietin antibody overnight at 4°C. 2) The following day the plates were washed 3 times with 300ul PBS

containing 0.15% Tween 20 (Sigma, Catalogue # P-9416). 3) The plates were then blocked with 300ul of 1% Bovine Serum Albumin (BSA; Sigma Catalogue # A-7030) in PBS for 1 hour at 37°C. 4) Repeat the washes as in step 2. 5) The clonal supernatants (75ul per clone per well of 96-well plate) were then added to the plate and incubated for 1 hour at 37°C. The clonal supernatant analyzed in the ELISA assay had been maintained on the cells 7 days prior to analysis. 6) Repeat the washes of step 2. 7) Add 75ul of polyclonal anti-human erythropoietin antibody (1/250 dilution in dilution buffer (0.5% BSA, 0.01% Tween 20, 1X PBS, pH 7.4) and incubate 1 hour at 37°C. 8) Repeat washes of step 2. 9) Add 75ul of goat anti-rabbit conjugated alkaline phosphatase diluted 1/4000 in dilution buffer and incubate 1 hour at 37°C. 10) Repeat washes of step 2. 11) Add 75ul substrate, p-nitrophenyl phosphate (Sigma N2640), diluted to 1mg/ml in substrate buffer (0.1 Ethanolamine-HCl (Sigma, Catalogue # E-6133), 5mM MgCl2 (Sigma, Catalogue # M-2393), pH 9.8). Incubate the plates in the dark for 1 hour at room temperature (22°C). 12) Read the absorption at 405nm (reference wavelength 495nm) on an Universal Microplate Reader (Bio-Tek Instruments, Inc., model # ELX800 UV). The erythropoietin standard curve was derived from readings of diluted human recombinant Erythropoietin (Roche, catalogue # 1-120-166; dilution range 125 - 7.8mUnits/ml). From this preliminary assay the 21 clones displaying the highest expression of erythropoietin were analyzed a second time in the same manner using medium supernatants that had been on the clones for 24 hours and a 1:3 dilution therof.

2. PCR Analysis

Genomic DNA was isolated from the 21 clones with the best expression (as assessed by the initial ELISA assay above) as well as the B19-38 cell line and used for PCR analysis. Genomic DNA was isolated using the Wizard genomic DNA purification kit (Promega) according to the

manufacturers protocol. Amplification was performed on 100ng of genomic DNA as template with MasterTaq DNA Polymerase (Eppendorf) and the primer set 5PacSV40 – CTGTTAATTAACTGTGGAATGTGTG TCAGTTAGGGTG (SEQ ID NO: 76) and Antisense Zeo - TGAACAGGGTCACGTCGTCC (SEQ ID NO: 77). The amplification conditions were as follows: heat denaturation for 3 minutes (95°C); 30 cycles of a 30 second denaturation (95°C), 30 seconds annealing (from 65oC decreasing 0.5oC per cycle to 50°C), and 1 minutes extension (72°C); 15 cycles of a 30 second denaturation (95°C), 30 seconds annealing (50°C), and 1 minute extension (72°C); the last cycle is followed by a 10 minute extension at 72°C. PCR products were size separated by gel electrophoresis. Of the 21 clones analyzed 19 produced a PCR product of 650 bp as expected for a site-specific integration event. All nineteen clones were the result of transformations with p19EPOcDNA (5) or p18genEPO (14) and pCXLamIntR (i.e. mutant integrase). The remaining two clones, both of which were the result of transformation with p18genEPO (SEQ ID NO: 125) and pCXLamInt (i.e. wildtype integrase; SEQ ID NO: 127), produced a 400 bp PCR product.

Example 12

Preparation of a Transformation Vector Useful for the Induction of Plant Artificial Chromosome Formation

Plant artificial chromosomes (PACs) can be generated by introducing nucleic acid, such as DNA, which can include a targeting DNA, for example rDNA or lambda DNA, into a plant cell, allowing the cell to grow, and then identifying from among the resulting cells those that include a chromosome with a structure that is distinct from that of any chromosome that existed in the cell prior to introduction of the nucleic acid. The structure of a PAC reflects amplification of chromosomal DNA, for example, segmented, repeat region-containing and heterochromatic

structures. It is also possible to select cells that contain structures that are precursors to PACs, for example, chromosomes containing more than one centromere and/or fragments thereof, and culture and/or manipulate them to ultimately generate a PAC within the cell.

In the method of generating PACs, the nucleic acid can be introduced into a variety of plant cells. The nucleic acid can include targeting DNA and/or a plant expressable DNA encoding one or multiple selectable markers (*e.g.*, DNA encoding bialophos (bar) resistance) or scorable markers (*e.g.*, DNA encoding GFP). Examples of targeting DNA include, but are not limited to, *N. tabacum* rDNA intergenic spacer sequence (IGS) and *Arabidopsis* rDNA such as the 18S, 5.8S, 26S rDNA and/or the intergenic spacer sequence. The DNA can be introduced using a variety of methods, including, but not limited to *Agrobacterium*-mediated methods, PEG-mediated DNA uptake and electroporation using, for example, standard procedures according to Hartmann *et al* [(1998) *Plant Molecular Biology 36*:741]. The cell into which such DNA is introduced can be grown under selective conditions and can initially be grown under non-selective conditions and then transferred to selective media. The cells or protoplasts can be placed on plates containing a selection agent to grow, for example, individual calli. Resistant calli can be scored for scorable marker expression. Metaphase spreads of resistance cultures can be prepared, and the metaphase chromosomes examined by FISH analysis using specific probes in order to detect amplification of regions of the chromosomes. Cells that have artificial chromosomes with functioning centromeres or artificial chromosomal intermediate structures, including, but not limited to, dicentric chromosomes, formerly dicentric chromosomes, minichromosomes, heterochromatin structures (e.g. sausage chromosomes), and stable self-replicating artificial chromosomal intermediates as described herein, are

identified and cultured. In particular, the cells containing self-replicating artificial chromosomes are identified.

The DNA introduced into a plant cell for the generation of PACs can be in any form, including in the form of a vector. An exemplary vector for use in methods of generating PACs can be prepared as follows.

For the production of artificial chromosomes, plant transformation vectors, as exemplified by pAgIIa and pAgIIb, containing a selectable marker, a targeting sequence, and a scorable marker were constructed using procedures well known in the art to combine the various fragments. The vectors can be prepared using vector pAg1 as a base vector and inserting the following DNA fragments into pAg1: DNA encoding β-glucoronidase under the control of the nopaline synthase (NOS) promoter fragment and flanked at the 3' end by the NOS terminator fragment, a fragment of mouse satellite DNA and an *N. tabacum* rDNA intergenic spacer sequence (IGS). In constructing plant transformation vectors, vector pAg2 can also be used as the base vector.

1. Construction of pAG1

Vector pAg1 (SEQ. ID. NO: 89) is a derivative of the CAMBIA vector named pCambia 3300 (Center for the Application of Molecular Biology to International Agriculture, i.e., CAMBIA, Canberra, Australia; www.cambia.org), which is a modified version of vector pCambia 1300 to which has been added DNA from the bar gene confering resistance to phosphinothricin. The nucleotide sequence of pCambia 3300 is provided in SEQ. ID. NO: 90. pCambia 3300 also contains a lacZ alpha sequence containing a polylinker region.

pAg1 was constructed by inserting two new functional DNA fragments into the polylinker of pCambia 3300: one sequence containing an attB site and a promoterless zeomycin resistance-encoding DNA flanked at the 3' end by a SV40 polyA signal sequence, and a second

sequence containing DNA from the hygromycin resistance gene (hygromycin phosphotransferase) confering resistance to hygromycin for selection in plants. Although the zeomycin-SV40 polyA signal fusion is not expected to function in plant cells, it can be activated in mammalian cells by insertion of a functional promoter element into the attB site by site-specific recombination catalyzed by the Lambda att integrase. Thus, the inclusion of the attB-zeomycin sequences allows for evaluation of functionality of plant artificial chromosomes in mammalian cells by activation of the zeomycin resistance-encoding DNA, and provides an att site for further insertion of new DNA sequences into plant artificial chromosomes formed as a result of using pAg1 for plant transformation. The second functional DNA fragment allows for selection of plant cells with hygromycin. Thus, pAg1 contains DNA from the bar gene confering resisance to phosphinothricin, DNA from the hygromycin resistance gene, both resistance-encoding DNAs under the control of a separate cauliflower mosaic virus (CaMV) 35S promoter, and the attB-promoterless zeomycin resistance-encoding DNA.

pAg1 is a binary vector containing *Agrobacterium* right and left T-DNA border sequences for use in *Agrobacterium*-mediated transformation of plant cells or protoplasts with the DNA located between the border sequences. pAg1 also contains the pBR322 Ori for replication in *E.coli*. pAg1 was constructed by ligating *Hind*III/*Pst*I-digested p3300attBZeo with *Hind*III/*Pst*I-digested pBSCaMV35SHyg as follows.

a. Generation of p3300attBZeo

Plasmid pCambia 3300 was digested with *Pst*I/*Ecl*136 II and ligated with *Pst*I/*Stu*I-digested pLITattBZeo (the nucleotide sequence of pLITattBZeo is provided in SEQ. ID. NO: 91. (containing DNA encoding the zeocin resistance gene and an attB Integrase recognition sequence) to generate p3300attBZeo which contains an attB site, a promoterless

zeomycin resistance-encoding DNA flanked at the 3′ end by a SV40 polyA signal, and a reconstructed *Pst*I site.

b. Generation of pBSCaMV35SHyg

A DNA fragment containing DNA encoding hygromycin phosphotransferase flanked by the CaMV 35S promoter and the CaMV 35S polyA signal sequence was obtained by PCR amplification of plasmid pCambia 1302 (GenBank Accession No. AF234298 and SEQ. ID. NO: 92). The primers used in the amplification reaction were as follows:

CaMV35SpolyA:

5′-CTGAATTAACGCCGAATTAATTCGGGGGATCTG-3′ SEQ. ID. NO: 93

CaMV35Spr:

5′-CTAGAGCAGCTTGCCAACATGGTGGAGCA-3′ SEQ. ID. NO: 94

The 2100-bp PCR fragment was ligated with *Eco*RV-digested pBluescript II SK+ (Stratagene, La Jolla, CA, U.S.A.) to generate pBSCaMV35SHyg.

c. Generation of pAg1

To generate pAg1, pBSCaMV35SHyg was digested with *Hind*III/*Pst*I and ligated with *Hind*III/*Pst*I-digested p3300attBZeo. Thus, pAg1 contains the pCambia 3300 backbone with DNA conferring resistance to phophinothricin and hygromycin under the control of separate CaMV 35S promoters, an attB-promoterless zeomycin resistance-encoding DNA recombination cassette and unique sites for adding additional markers, *e.g.*, DNA encoding GFP. The attB site can be used as decribed hereing for the addition of new DNA sequences to plant artificial chromosomes, including PACs formed as a result of using the pAg1 vector, or derivatives thereof, in the production of PACs. The attB site provides a convenient site for recombinase-mediated insertion of DNAs containing a homologous att site.

2. pAG2

The vector pAg2 (SEQ. ID. NO: 95) is a derivative of vector pAg1 formed by adding DNA encoding a green fluorescent protein (GFP), under the control of a NOS promoter and flanked at the 3′ end by a NOS polyA signal, to pAg1. pAg2 was constructed as follows. A DNA fragment containing the NOS promoter was obtained by digestion of pGEM-T-NOS, or pGEMEasyNOS (SEQ. ID. NO: 96), containing the NOS promoter in the cloning vector pGEM-T-Easy (Promega Biotech, Madison, WI, U.S.A.), with *Xba*I/*Nco*I and was ligated to an *Xba*I/*Nco*I fragment of pCambia 1302 containing DNA encoding GFP (without the CaMV 35S promoter) to generate p1302NOS (SEQ. ID. NO: 97) containing GFP-encoding DNA in operable association with the NOS promoter. Plasmid p1302NOS was digested with *Sma*I/*Bsi*WI to yield a fragment containing the NOS promoter and GFP-encoding DNA. The fragment was ligated with *Pme*I/*Bsi*WI-digested pAg1 to generate pAg2. Thus, pAg2 contains DNA from the bar gene confering resistance to phosphinothricin, DNA conferring resistance to hygromycin, both resistance-encoding DNAs under the control of a cauliflower mosaic virus 35S promoter, DNA encoding kanamycin resistance, a GFP gene under the control of a NOS promoter and the attB-zeomycin resistance-encoding DNA. One of skill in the art will appreciate that other fragments can be used to generate the pAg1 and pAg2 derivatives and that other heterlogous DNA can be incorporated into pAg1 and pAg2 derivatives using methods well known in the art.

3. pAgIIa and pAgIIb transformation vectors

Vectors pAgIIa and pAgIIb were constructed by inserting the following DNA fragments into pAg1: DNA encoding *β*-glucoronidase, the nopaline synthase terminator fragment, the nopaline synthase (NOS) promoter fragment, a fragment of mouse satellite DNA and an *N. tabacum*

rDNA intergenic spacer sequence (IGS). The construction of pAgIIa and pAgIIb was as follows.

An *N. tabacum* rDNA intergenic spacer (IGS) sequence (SEQ. ID. NO: 98; see also GenBank Accession No. Y08422; see also Borysyuk *et al.* (2000) *Nature Biotechnology 18*:1303-1306; Borysyuk *et al.* (1997) *Plant Mol. Biol.35*:655-660; U.S. Patent Nos. 6,100,092 and 6,355,860) was obtained by PCR amplification of tobacco genomic DNA. The IGS can be used as a targeting sequence by virtue of its homology to tobacco rDNA genes; the sequence is also an amplification promoter sequence in plants. This fragment was amplified using standard PCR conditions (*e.g.*, as described by Promega Biotech, Madison, WI, U.S.A.) from tobacco genomic DNA using the primers shown below:

NTIGS-FI

5'- GTG CTA GCC AAT GTT TAA CAA GAT G- 3' (SEQ ID No. 99) and

NTIGS-RI

5'-ATG TCT TAA AAA AAA AAA CCC AAG TGA C- 3' (SEQ ID No. 100)

Following amplification, the fragment was cloned into pGEM-T Easy to give pIGS-I A fragment of mouse satellite DNA (Msat1 fragment; GenBank Accession No. V00846; and SEQ ID No. 101) was amplified via PCR from pSAT-1 using the following primers:

MSAT-F1

5'- AAT ACC GCG GAA GCT TGA CCT GGA ATA TCG C -3'(SEQ ID No. 102) and

MSAT-Ri

5'-ATA ACC GCG GAG TCC TTC AGT GTG CA T- 3' (SEQ ID No. 103)

This amplification added a *Sac*II and a *Hind*III site at the 5'end and a *Sac*II site at the 3' end of the PCR fragment. This fragment was then cloned into the *Sac*II site in pIGS-1 to give pMIGS-1, providing a eukaryotic

centromere-specific DNA and a convenient DNA sequence for detection via FISH.

A functional marker gene containing a NOS-promoter:GUS:NOS terminator fusion was then constructed containing the NOS promoter (GenBank Accession No. U09365; SEQ ID No. 104), *E. coli* β-glucuronidase coding sequence (from the GUS gene; GenBank Accession No. S69414; and SEQ ID No. 105), and the nopaline synthase terminator sequence (GenBank Accession No. U09365; SEQ ID No. 107). The NOS promoter in pGEM-T-NOS was added to a promoterless GUS gene in pBlueScript (Stratagene, La Jolla, CA, U.S.A.) using *Not*I/*Spe*I to form pNGN-1, which has the NOS promoter in the opposite orientation relative to the GUS gene.

pMIGS-1 was digested with *Not*I/*Spe*I to yield a fragment containing the mouse major satellite DNA and the tobacco IGS which was then added to *Not*I-digested pNGN-1 to yield pNGN-2. The NOS promoter was then re-oriented to provide a functional GUS gene, yielding pNGN-3, by digestion and religation with *Spe*I. Plasmid pNGN-3 was then digested with *Hind*III, and the *Hind*III fragment containing the β-glucuronidase coding sequence and the rDNA intergenic spacer, along with the Msat sequence, was added to pAG-1 to form pAgIIa (SEQ ID NO: 108), using the unique *Hind*III site in pAg1 located near the right T-DNA border of pAg1, within the T-DNA region.

Another plasmid vector, referred to as pAgIIb, was also recovered, which contained the inserted HindIII fragment (SEQ ID NO: 108) in the opposite orientation relative to that observed in pAgIIa. Thus, pAgIIa and pAgIIb differ only in the orientation of the HindIII fragment containing the mouse major satellite sequence, the GUS DNA sequence and the IGS sequence. The nucleotide sequences of pAgIIa is provided in SEQ. ID. NOS: 109.

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A eukaryotic chromosome comprising one or a plurality of *att* site(s), wherein:

an *att* site is heterologous to the chromosome; and

an *att* site permits site-directed integration in the presence of lambda integrase.

2. The eukaryotic chromosome of claim 1, wherein the *att* sites are selected from the group consisting of *att*P and *att*B or *att*L and *att*R, or variants thereof.

3. The eukaryotic chromosome of claim 1 that is an artificial chromosome.

4. The eukaryotic chromosome of claim 1 that is an artificial chromosome expression system (*ACes*).

5. The eukaryotic chromosome of claim 4 that is predominantly heterochromatin.

6. The chromosome of claim 1 that is an artificial chromosome that contains no more than about 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% euchromatin.

7. The chromosome of claim 1 that is a plant chromosome.

8. The chromosome of claim 1 that is an animal chromosome.

9. The chromosome of claim 7 that is a plant artificial chromosome.

10. The chromosome of claim 8 that is an animal artificial chromosome.

11. The chromosome of claim 8 that is a mammalian chromosome.

12. The chromosome of claim 11 that is a mammalian artificial chromosome.

13. The chromosome of claim 6 that is an artificial chromosome expression system (*ACes*).

14. A platform artificial chromosome expression system (*ACes*) comprising one or a plurality of sites that participate in recombinase catalyzed recombination.

15. The *ACes* of claim 14 that contains one site.

16. The *ACes* of claim 14 that is predominantly heterochromatin.

17. The *ACes* of claim 14 that contains no more than about 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% euchromatin.

18. The *ACes* of claim 14 that is a plant *ACes*.

19. The *ACes* of claim 14 that is an animal *ACes*.

20. The *ACes* of claim 14 that is selected from a fish, insect, reptile, amphibian, arachnid or a mammalian *ACes*.

21. The *ACes* of claim 14 that is a fish *ACes*.

22. The artificial chromosome expression system (*ACes*) of claim 14, wherein the recombinase and site(s) are from the Cre/lox system of bacteriophage P1, the int/att system of lambda phage, the FLP/FRT system of yeast, the Gin/gix recombinase system of phage Mu, the Cin recombinase system, the Pin recombinase system of *E. coli* and the R/RS system of the pSR1 plasmid, or any combination thereof.

23. A method of introducing heterologous nucleic acid into a chromosome, comprising:

contacting a chromosome of any of claims 1 or 14 with a nucleic acid molecule comprising both the heterologous nucleic acid and a recombination site, in the presence of a recombinase that promotes recombination between the sites in the chromosome and in the nucleic acid molecule.

24. The method of claim 23, wherein the recombinase is selected from the group consisting of Cre, Gin, Cin, Pin, FLP, a phage integrase and R from the pSR1 plasmid.

25. The method of claim 23, wherein the nucleic acid molecule encodes a therapeutic protein, antisense nucleic acid, or comprises an artificial chromosome.

26. The method of claim 25, wherein the nucleic acid molecule comprises a yeast artificial chromosomes (YAC), a bacterial artificial chromosome (BAC) or an insect artificial chromosome (IAC).

27. A combination, comprising, the chromosome of claim 1 and a first vector comprising the cognate recombination site, wherein the cognate recombination site is a site that recombines with the site engineered into the chromosome.

28. The combination of claim 27, further comprising nucleic acid encoding a recombinase, wherein the nucleic acid is on a second vector or on the first vector, or on the *ACes* under an inducible promoter.

29. The combination of claim 28, wherein the recombinase and sites are from the Cre/lox system of bacteriophage P1, the int/att system of lambda phage, the FLP/FRT system of yeast, the Gin/gix recombinase system of phage Mu, the Pin recombinase system of *E. coli* and the R/RS system of the pSR1 plasmid, or any combination thereof.

30. The combination of claim 28, wherein a vector is the plasmid pCXLamIntR.

31. The combination of claim 27, wherein a vector is the plasmid pDsRedN1-attB.

32. A kit, comprising the combination of claim 27 and optionally instructions for introducing heterologous nucleic acid into the chromosome.

33. A method for introducing heterologous nucleic acid into a platform artificial chromosome, comprising:

(a) mixing an artificial chromosome comprising at least a first recombination site and a vector comprising at least a second recombination site and the heterologous nucleic acid;

(b) incubating the resulting mixture in the presence of at least one recombination protein under conditions whereby recombination between the first and second recombination sites is effected, thereby introducing the heterologous nucleic acid into the artificial chromosome.

34. The method of claim 33, wherein the artificial chromosome is an *ACes*.

35. The method of claim 33, wherein said mixing step (a) is conducted in cells ex vivo.

36. The method of claim 33, wherein said mixing step (a) is conducted extracellularly in an in vitro reaction mixture.

37. The method of claim 33, wherein the at least one recombination protein is encoded by a bacteriophage selected from the group consisting of bacteriophage lambda, phi 80, P22, P2, 186, P4 and P1.

38. The method of claim 37, wherein the at least one recombination protein is encoded by bacteriophage lambda, or mutants thereof.

39. The method of claim 33, wherein at least one recombination protein is selected from the group consisting of Int, IHF, Xis and Cre, $\gamma\delta$, Tn3 resolvase, Hin, Gin, Cin and Flp.

40. The method of claim 32, wherein the recombination sites are selected from the group consisting of att and lox P sites.

41. The method of claim 33, wherein the first and/or second recombination site contains at least one mutation that removes one or more stop codons.

42. The method of claim 33, wherein the first and/or second recombination site contains at least one mutation that avoids hairpin formation.

43. The method of claim 33, wherein the first and/or second recombination site comprises at least a first nucleic acid sequence selected from the group consisting of SEQ ID NOs:41-56:

a) RKYCWGCTTTYKTRTACNAASTSGB (m-att) (SEQ ID NO:41);

b) AGCCWGCTTTYKTRTACNAACTSGB (m-attB) (SEQ ID NO:42);

c) GTTCAGCTTTCKTRTACNAACTSGB (m-attR) (SEQ ID NO:43);

d) AGCCWGCTTTCKTRTACNAAGTSGB (m-attL) (SEQ ID NO:44);

e) GTTCAGCTTTYKTRTACNAAGTSGB (m-attP1) (SEQ ID NO:45);

f) AGCCTGCTTTTTTGTACAAACTTGT (attB1) (SEQ ID NO:46);

g) AGCCTGCTTTCTTGTACAAACTTGT (attB2) (SEQ ID NO:47);

h) ACCCAGCTTTCTTGTACAAACTTGT (attB3) (SEQ ID NO:48);

i) GTTCAGCTTTTTTGTACAAACTTGT (attR1) (SEQ ID NO:49);

j) GTTCAGCTTTCTTGTACAAACTTGT (attR2) (SEQ ID NO:50);

k) GTTCAGCTTTCTTGTACAAAGTTGG (attR3) (SEQ ID NO:51);

l) AGCCTGCTTTTTTGTACAAAGTTGG (attL1) (SEQ ID NO:52);

m) AGCCTGCTTTCTTGTACAAAGTTGG (attL2) (SEQ ID NO:53);

n) ACCCAGCTTTCTTGTACAAAGTTGG (attL3) (SEQ ID NO:54);

o) GTTCAGCTTTTTTGTACAAAGTTGG (attP1) (SEQ ID NO:55);

p) GTTCAGCTTTCTTGTACAAAGTTGG (attP2, P3) (SEQ ID NO: 56);

and a corresponding or complementary DNA or RNA sequence, wherein R=A or G, K=G or T/U, Y=C or T/U, W=A or T/U, N=A or C or G or T/U, S=C or G, and B=C or G or T/U; and

the core region does not contain a stop codon in one or more reading frames.

44. The method of claim 33, wherein the first and/or second recombination site comprises at least a first nucleic acid sequence selected from the group consisting of a mutated att recombination site containing at least one mutation that enhances recombinational specificity, a complementary DNA sequence thereto, and an RNA sequence corresponding thereto.

45. The method of claim 33, wherein the vector comprising the second site further encodes at least one selectable marker.

46. The method of claim 45, wherein the marker is a promoterless marker, which, upon recombination is under the control of a promoter and is thereby expressed.

47. The method of claim 46, wherein the first recombination site is attP and is in the sense orientation prior to recombination.

48. The method of claim 46, wherein the selectable marker is selected from the group consisting of an antibiotic resistance gene, and a detectable protein, wherein the detectable protein is chromogenic, fluorescent, or capable of being bound by an antibody and FACs sorted.

49. The method of claim 48, wherein the selectable marker is selected from the group consisting of green fluorescent protein (GFP), red fluorescent protein (RFP), blue fluorescent protein (BFP), and *E. coli* histidinol dehydrogenase (hisD).

50. A cell comprising, the chromosome of claim 1.

51. The cell of claim 50, wherein the cell is a nuclear donor cell.

52. The cell of claim 50, wherein the cell is a stem cell.

53. The stem cell of claim 52, wherein said stem cell is human and is selected from the group consisting of a mesenchymal stem cell, a hematopoietic stem cell, an adult stem cell and an embryonic stem cell.

54. The cell of claim 50, wherein the cell is mammalian.

55. The cell of claim 54, wherein the mammal is selected from the group consisting of humans, primates, cattle, pigs, rabbits, goats, sheep, mice, rats, guinea pigs, hamsters, cats, dogs, and horses.

56. The cell of claim 50, wherein the cell is a plant cell.

57. A cell comprising the platform *ACes* of claim 14.

58. The cell of claim 57, wherein the cell is a nuclear donor cell.

59. The cell of claim 57, wherein the cell is a stem cell.

60. The stem cell of claim 59, wherein said stem cell is human and is selected from the group consisting of a mesenchymal stem cell, a hematopoietic stem cell, an adult stem cell and an embryonic stem cell.

61. A human mesenchymal cell comprising an artificial chromosome.

62. The human mesenchymal cell of claim 61, wherein said artificial chromosome is an *ACes*.

63. The human mesenchymal cell of claim 62, wherein the *ACes* is a platform-*ACes*.

64. A method for introducing heterologous nucleic acid into the mesenchymal cell of claim 63, comprising:

(a) introducing into the cell of claim 63, wherein the platform-*ACes* has a first recombination site, a vector comprising at least a second recombination site and the heterologous nucleic acid;

(b) incubating the resulting mixture in the presence of at least one recombination protein under conditions whereby recombination between the first and second recombination sites is effected, thereby introducing the heterologous nucleic acid into the platform-*ACes* within the mesenchymal cell.

65. A lambda-intR mutein comprising a glutamic acid to arginine change at position 174 of wild-type lambda-intR.

66. The lambda-intR mutein of claim 65, wherein the lambda-intR mutein comprises SEQ ID NO:37.

67. The method of claim 46 wherein the promoterless marker is transcriptionally downstream of the heterologous nucleic acid, wherein the heterologous nucleic acid encodes a heterologous protein, and wherein the expression level of the selectable marker is transcriptionally linked to the expression level of the heterologous protein.

68. The method of claim 67, wherein the selectable marker and the heterologous nucleic acid are transcriptionally linked by the presence of a IRES between them.

69. The method of claim 68, wherein the selectable marker is selected from the group consisting of an antibiotic resistance gene, and a detectable protein, wherein the detectable protein is chromogenic or fluorescent.

70. The method of claim 69, wherein the selectable marker is selected from the group consisting of green fluorescent protein (GFP), red fluorescent protein (RFP), blue fluorescent protein (BFP), and *E. coli* histidinol dehydrogenase.

71. The method of claim 67 further comprising expressing the heterologous protein and isolating the heterologous protein.

72. A method for producing a transgenic animal, comprising introducing a platform-*ACes* into an embryonic cell.

73. The method of claim 72, wherein the embryonic cell is a stem cell.

74. The method of claim 72, wherein the embryonic cell is in an embryo.

75. The method of claim 72, wherein the platform-*ACes* comprises heterologous nucleic acid that encodes a therapeutic product.

76. The method of claim 72, wherein the transgenic animal is a fish, insect, reptile, amphibians, arachnid or mammal.

77. The method of claim 72, wherein the *ACes* is introduced by cell fusion, lipid-mediated transfection by a carrier system, microinjection, microcell fusion, electroporation, microprojectile bombardment or direct DNA transfer.

78. A transgenic animal produced by the method of claim 72.

79. A cell line useful for making a library of *ACes*, comprising a multiplicity of heterologous recombination sites randomly integrated throughout the endogenous chromosomes.

80. A method of making a library of *ACes* comprising random portions of a genome, comprising introducing one or more *ACes* into the cell line of claim 79, under conditions that promote the site-specific chromosomal arm exchange of the *ACes* into, and out of, a multiplicity of the heterologous recombination sites within the cell's chromosomal DNA; and isolating said multiplicity of *ACes*, thereby producing a library of *ACes* whereby multiple *ACes* have different portions of the genome within.

81. A library of cells useful for genomic screening, said library comprising a multiplicity of cells, wherein each cell comprises an *ACes* having a mutually exclusive portion of a chromosomal nucleic acid therein.

82. The library of cells of claim 81, wherein the cells of the library are from a different species than the chromosomal nucleic acid within the *ACes*.

83. A method of making one or more cell lines, comprising

a) integrating into endogenous chromosomal DNA of a selected cell species, a multiplicity of heterologous recombination sites,

b) introducing a multiplicity of *ACes* under conditions that promote the site-specific chromosomal arm exchange of the *ACes* into, and out of, a multiplicity of the heterologous recombination sites integrated within the cell's endogenous chromosomal DNA;

c) isolating said multiplicity of *ACes*, thereby producing a library of *ACes* whereby a multiplicity of *ACes* have mutually exclusive portions of the endogenous chromosomal DNA therein;

d) introducing the isolated multiplicity of *ACes* of step c) into a multiplicity of cells, thereby creating a library of cells;

e) selecting different cells having mutually exclusive *ACes* therein and clonally expanding or differentiating said different cells into clonal cell cultures, thereby creating one or more cell lines.

84. The method of claim 23, wherein the nucleic acid molecule with a recombination site is a PCR product.

85. Method of claim 23 wherein the recombinase is a protein and the recombination event occurs in vitro.

86. The method of claim 33, wherein the vector is a PCR *product comprising a second recombination site.*

87. The lambda-intR mutein of claim 65, wherein the mutein further comprises an amino acid signal for nuclear localization.

88. The lambda-intR mutein of claim 65, wherein the mutein further comprises an epitope tag for protein purification.

89. A modified iron-induced promoter comprising SEQ ID NO:128.

90. A plasmid or expression cassette comprising the promoter of claim 89.

91. A vector, comprising:

a recognition site for recombination; and

a sequence of nucleotides that targets the vector to an amplifiable region of a chromosome.

92. The vector of claim 91, wherein the amplifiable region comprises heterochromatic nucleic acid.

93. The vector of claim 91, wherein the amplifiable region comprises rDNA.

94. The vector of claim 93, wherein the rDNA comprises an intergenic spacer.

95. The vector of claim 91, further comprising nucleic acid encoding a selectable marker that is not operably associated with any promoter.

96. The vector of claim 91, wherein the chromosome is a mammalian chromosome.

97. The vector of claim 91, wherein the chromosome is a plant chromosome.

98. A cell of claim 57 that is a plant cell, wherein the *ACes* platform is a MAC.

99. The plant cell of claim 98, wherein the MAC comprises transcriptional regulatory sequence of nucleotides derived from plants.

100. The plant cell of claim 99, wherein the regulatory sequence is selected from the group consisting of promoters, terminators, enhancers, silencers and transcription factor binding sites.

101. A cell of claim 57 that is an animal cell, wherein the *ACes* platform is a plant artificial chromosome (PAC).

102. The cell of claim 101 that is a mammalian cell.

103. The cell of claim 98, wherein the MAC comprises transcriptional regulatory sequence of nucleotides derived from plants.

104. The cell of claim 102, wherein the MAC comprises transcriptional regulatory sequence of nucleotides derived from plants.

105. The cell of claim 104, wherein the regulatory sequence is selected from the group consisting of promoters, terminators, enhancers, silencers and transcription factor binding sites.

106. A method, comprising:

introducing a vector of claim 91 into a cell;

growing the cells; and

selecting a cell comprising an artificial chromosome that comprises one or more repeat regions.

107. The method of claim 106, wherein sufficient portion of the vector integrates into a chromosome in the cell to result in amplification of chromosomal DNA.

108. The method of claim 106, wherein the artificial chromsome is an *ACes*.

109. A method for screening, comprising:

contacting a cell comprising a reporter *ACes* with test compounds or known compounds, wherein:

the reporter *ACes* comprises one or a plurality of reporter constructs;

a reporter construct comprises a reporter gene in operative linkage with a regulatory region responsive to test or known compounds; and

detecting any increase or decrease in signal output from the reporter, wherein a change in the signal is indicative of activity of the test or known compound on the regulatory region.

110. The method of claim 109, wherein the reporter is operatively linked to a promoter that controls expression of a gene in a signal transduction pathway, whereby activation or reduction in the signal indicates that the pathway is activated or down-regulated by the test compound.

111. The method of claim 109, wherein the reporter in the construct encodes drug resistance or encodes a fluorescent protein.

112. The method of claim 111, wherein the fluorescent protein is selected from the group consisting of red, green and blue fluorescent proteins.

113. The method of claim 109, wherein the *ACes* comprises a plurality of reporter-linked constructs, each with a different reporter, whereby the pathway(s) affected by the test compounds can be elucidated.

114. The method of claim 109, wherein a reporter is operatively linked to a promoter that is transcriptionally regulated in resopnse to DNA damage, and the test compounds are genotoxicants.

115. The method of claim 114, wherein the DNA damage is induced by apoptosis, necrosis or cell-cycle perturbations.

116. The method of claim 114, wherein unknown compounds are screened to assess whether they are genotoxicants.

117. The method of claim 114, wherein the promoter is a cytochrome P450-profiled promoter.

118. The method of claim 114, wherein the cell is in a transgenic animal and toxicity is assessed in the animal.

119. The method of claim 109, wherein:

the cell is a patient cell sample; the patient has a disease;

the regulatory region is one targeted by a drug or drug regimen; and

the method assesses the effectiveness of a treatment for the disease for the particular patient.

120. The method of claim 119, wherein the cell is a tumor cell.

121. The method of claim 109, wherein the cell is a stem cell or a progenitor cell, whereby expression of the reporter is operatively linked to

a regulatory region exprssed in the cells to thereby identify stem cells or progenitor cell.

122. The method of claim 109, wherein the cell is in an animal; and the method comprises whole-body imaging to monitor expression of the reporter in the animal.

123. A reporter *ACes* comprises one or a plurality of reporter constructs, wherein the reporter construct comprises a reporter gene in operative linkage with a regulatory region responsive to test or known compounds.

Fig. 1 Generation of *ACes* for Platform Chromosome Engineering

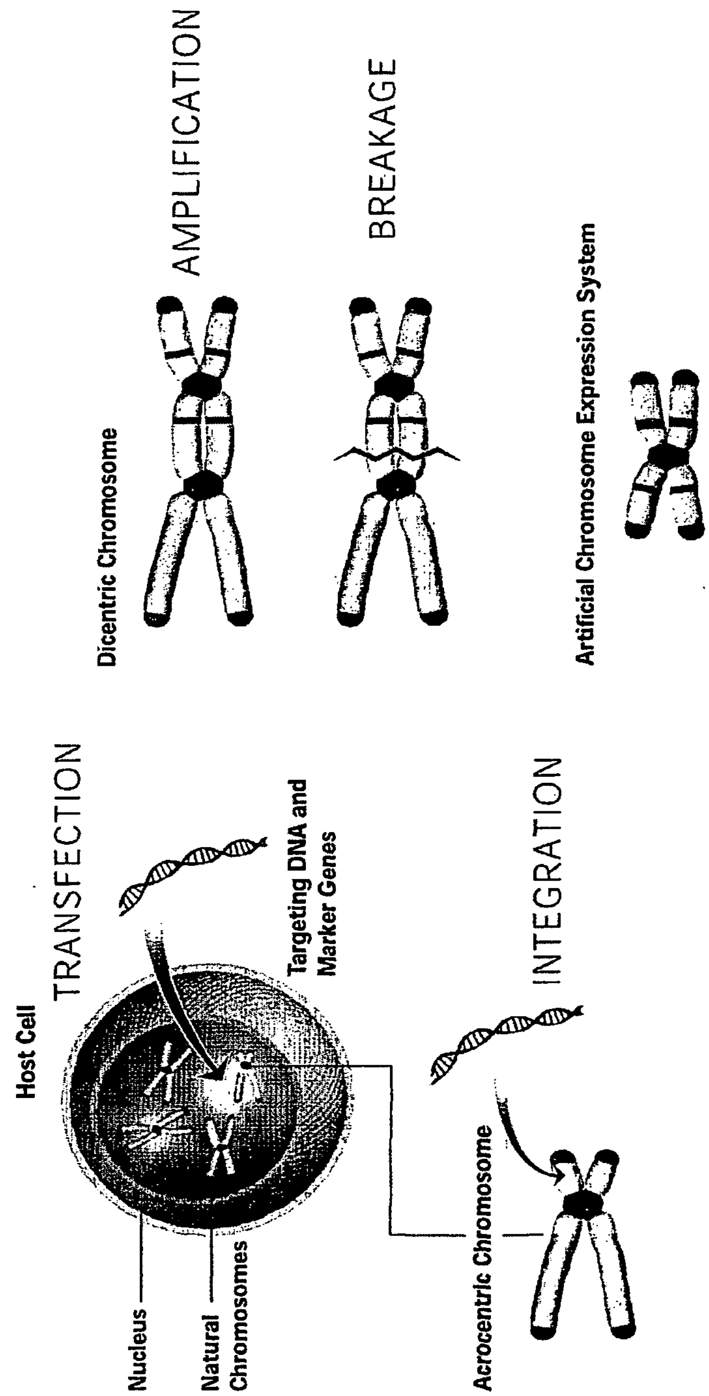


Fig. 2 Targeting plasmid pWEPuro9K

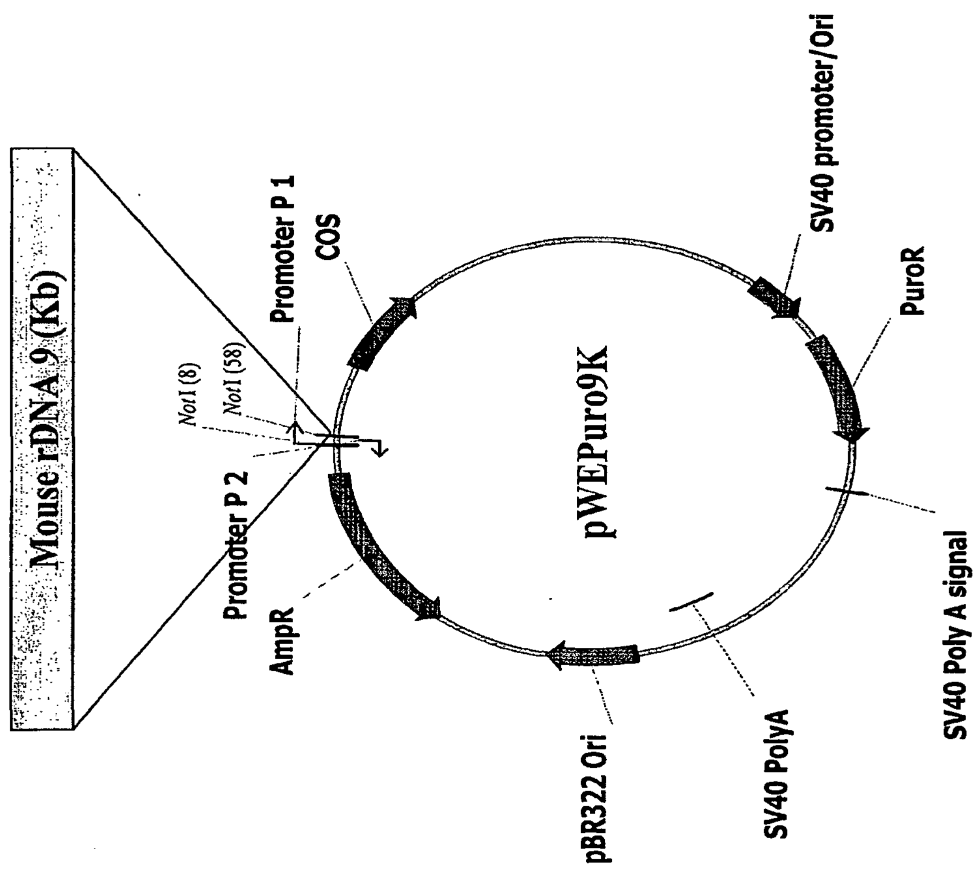
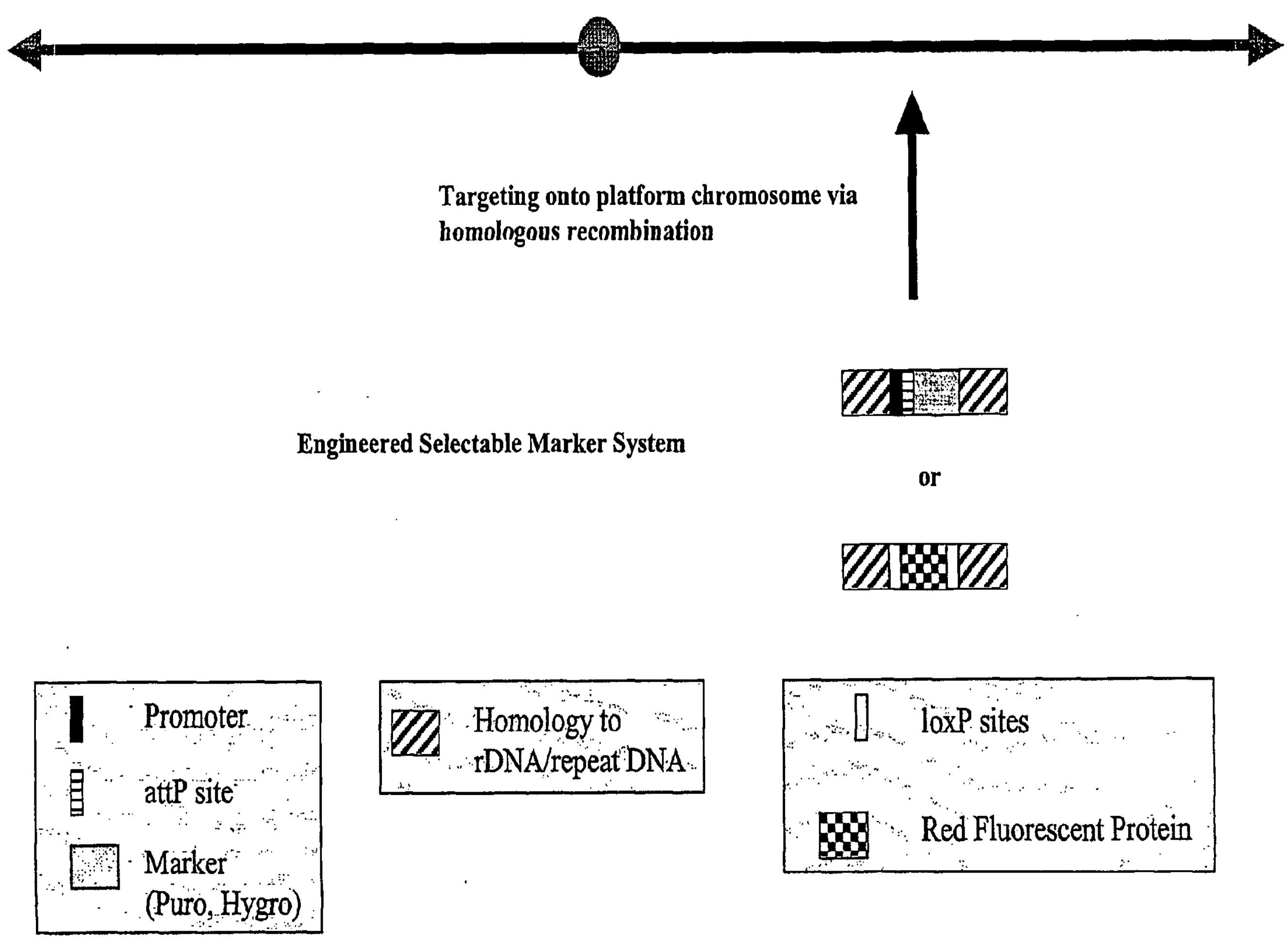


Fig. 3 Platform Chromosome with single recombination site

Platform chromosome containing mouse rDNA, mouse major/minor repeat DNA and puromycin resistant cassette



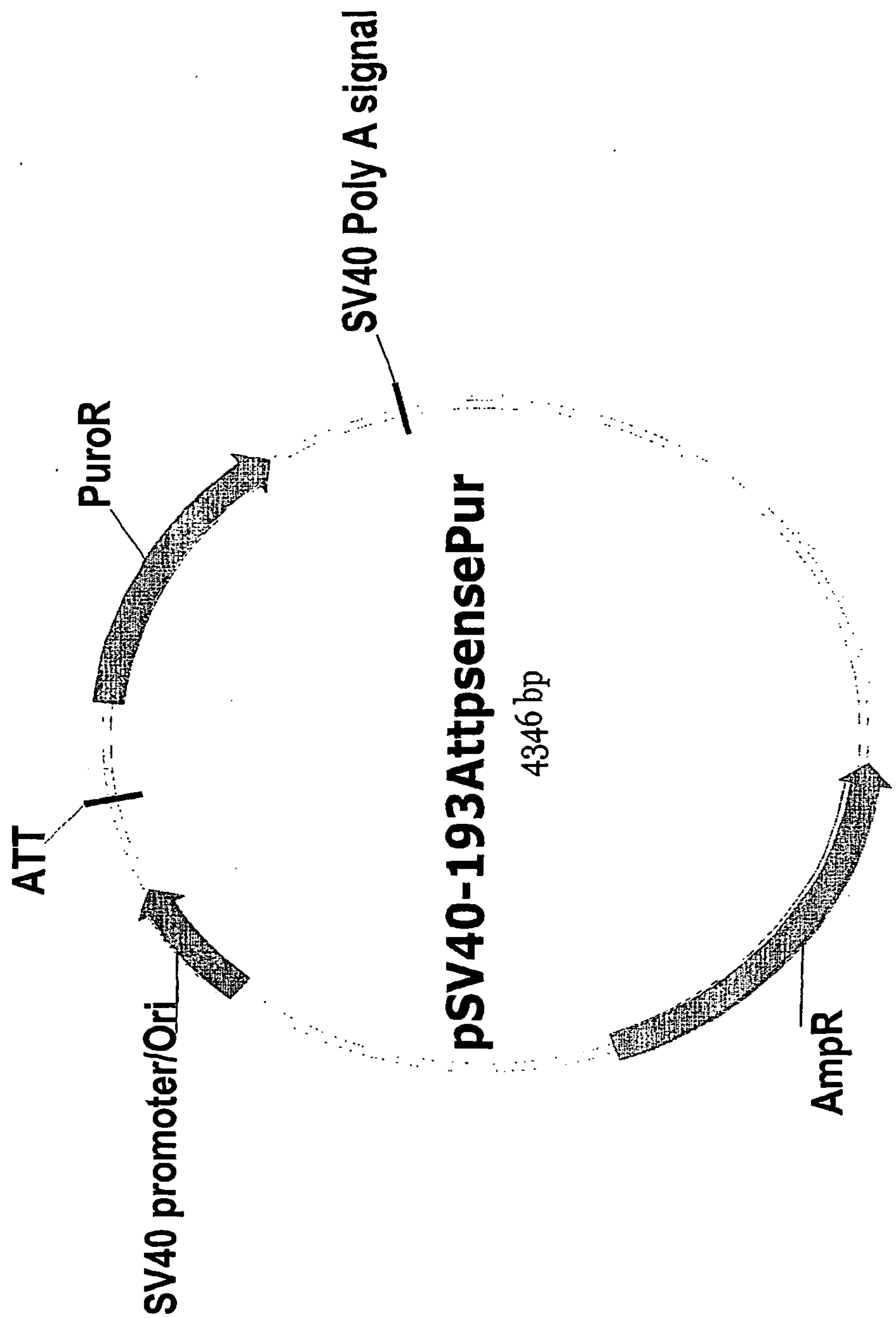

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Fig. 4

Fig. 5 Generation of chromosome based platform with multiple integration sites

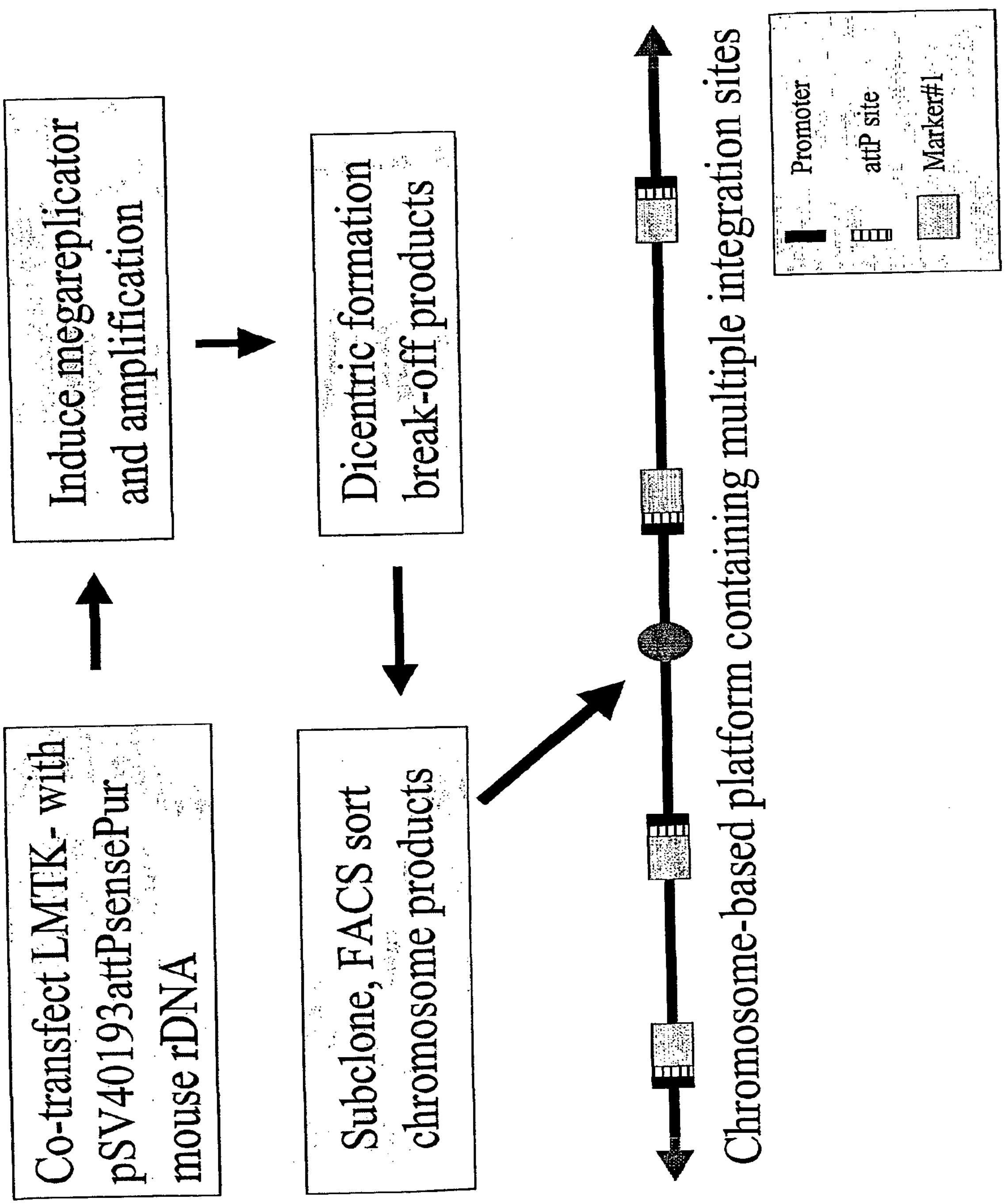

Fig. 6 λ integrase recombination

attP x attB $\rightleftarrows$ attL x attR

	Core Region
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attL	CTGCTTTTTTATACTAAGTTG
attR	CAGCTTTTTTATACTAACTTG

Fig. 7 λINT recombination on artificial chromosome

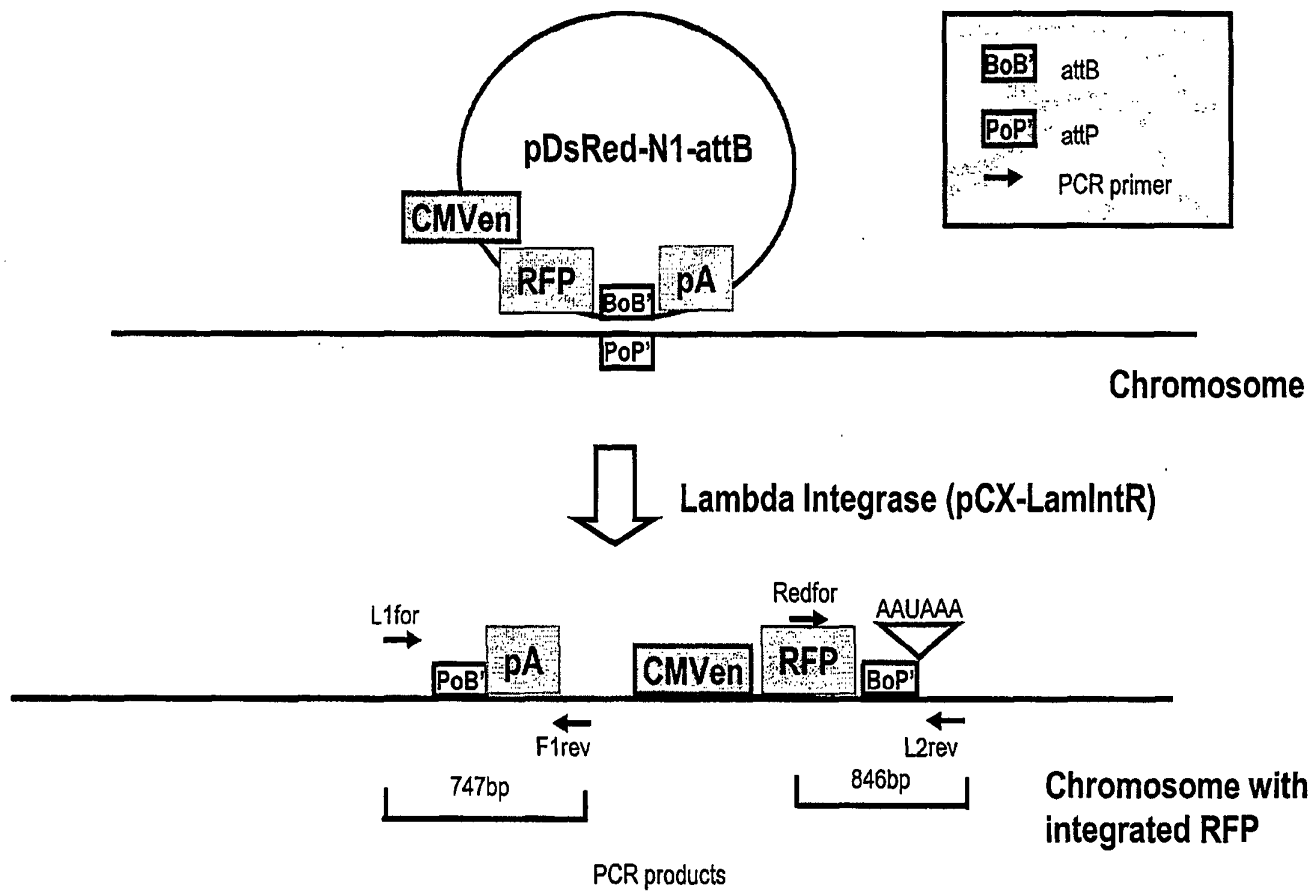


Fig. 8 pCXLamIntR integrase expression vector

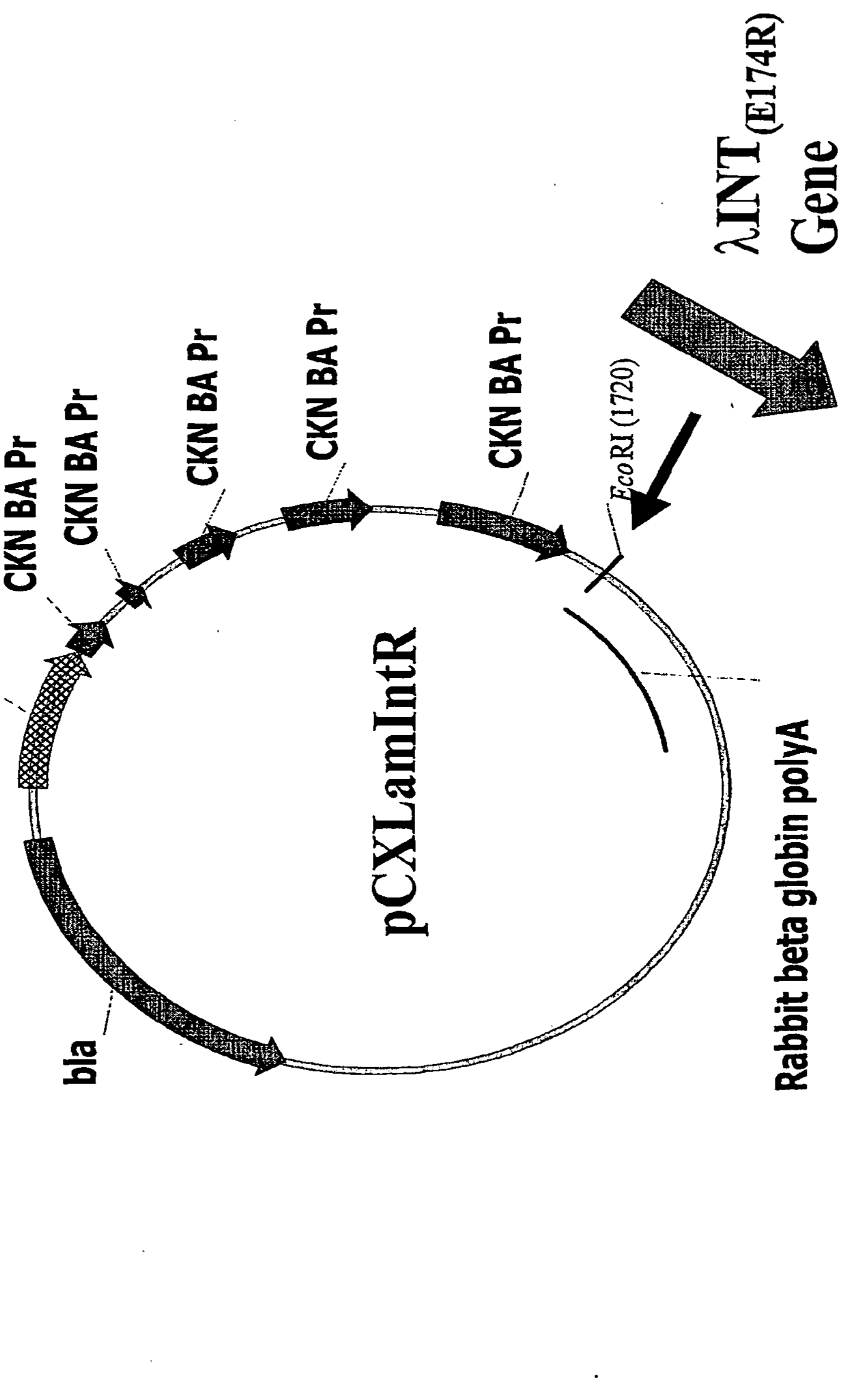
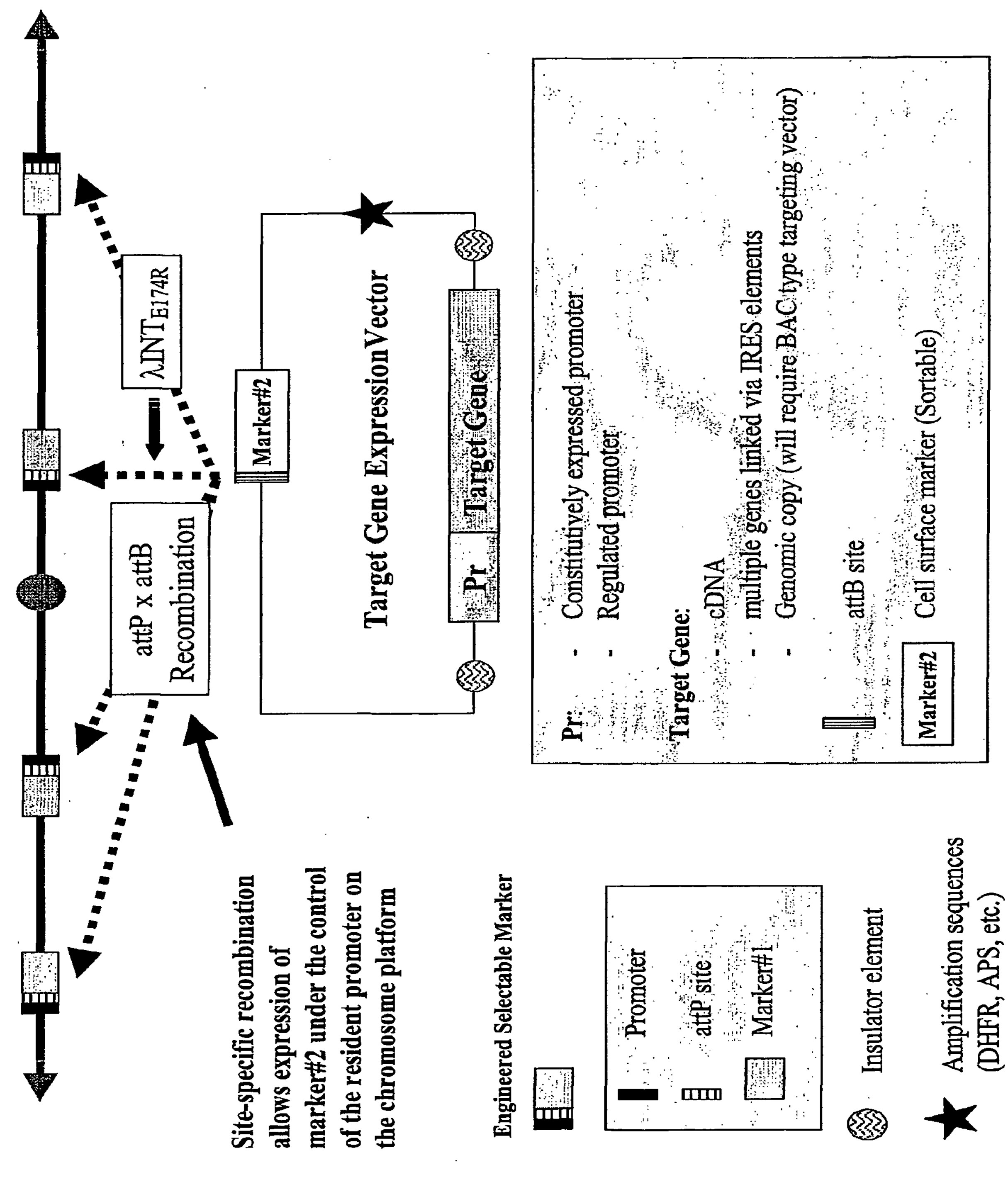


Fig. 9 Platform Technology



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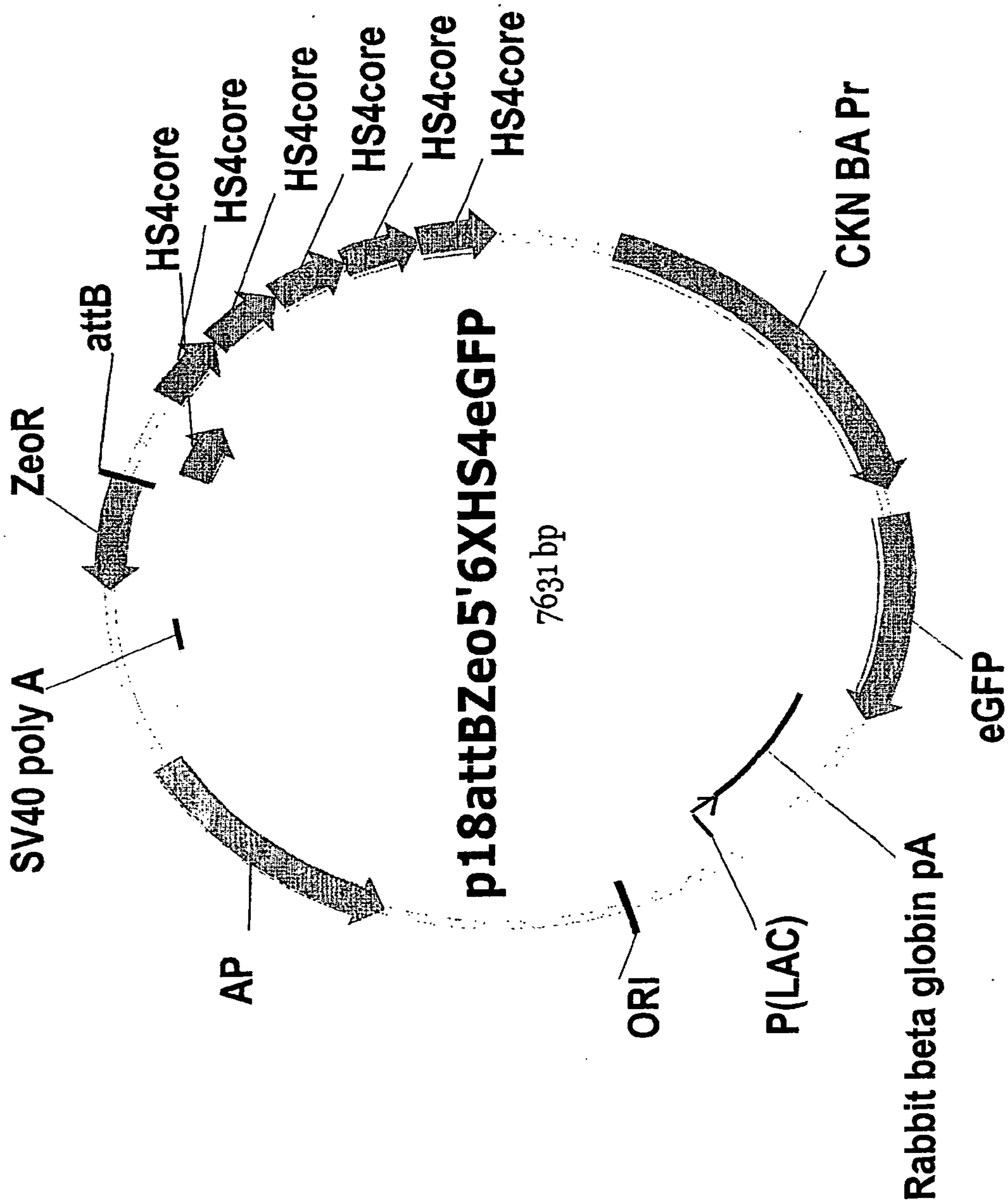
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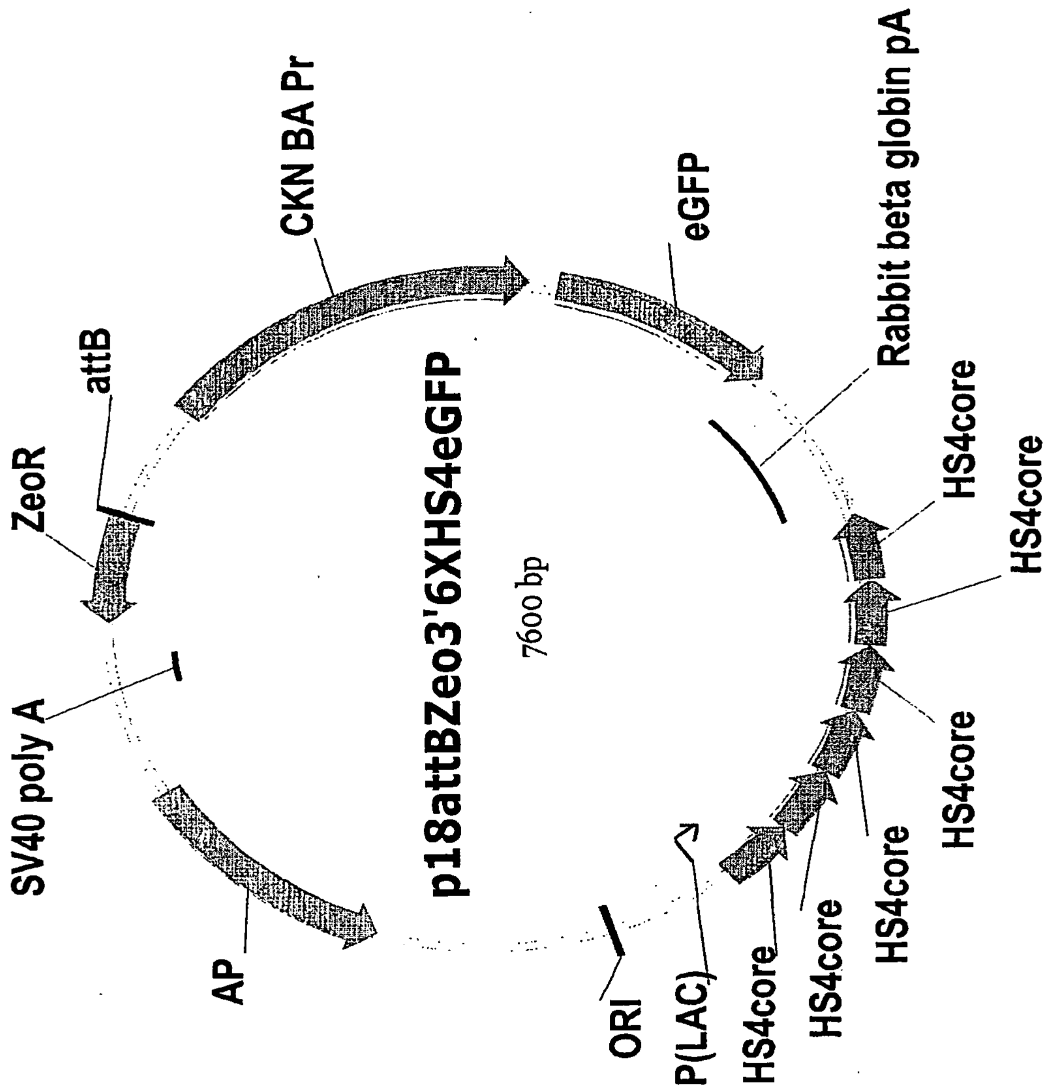
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Fig. 11

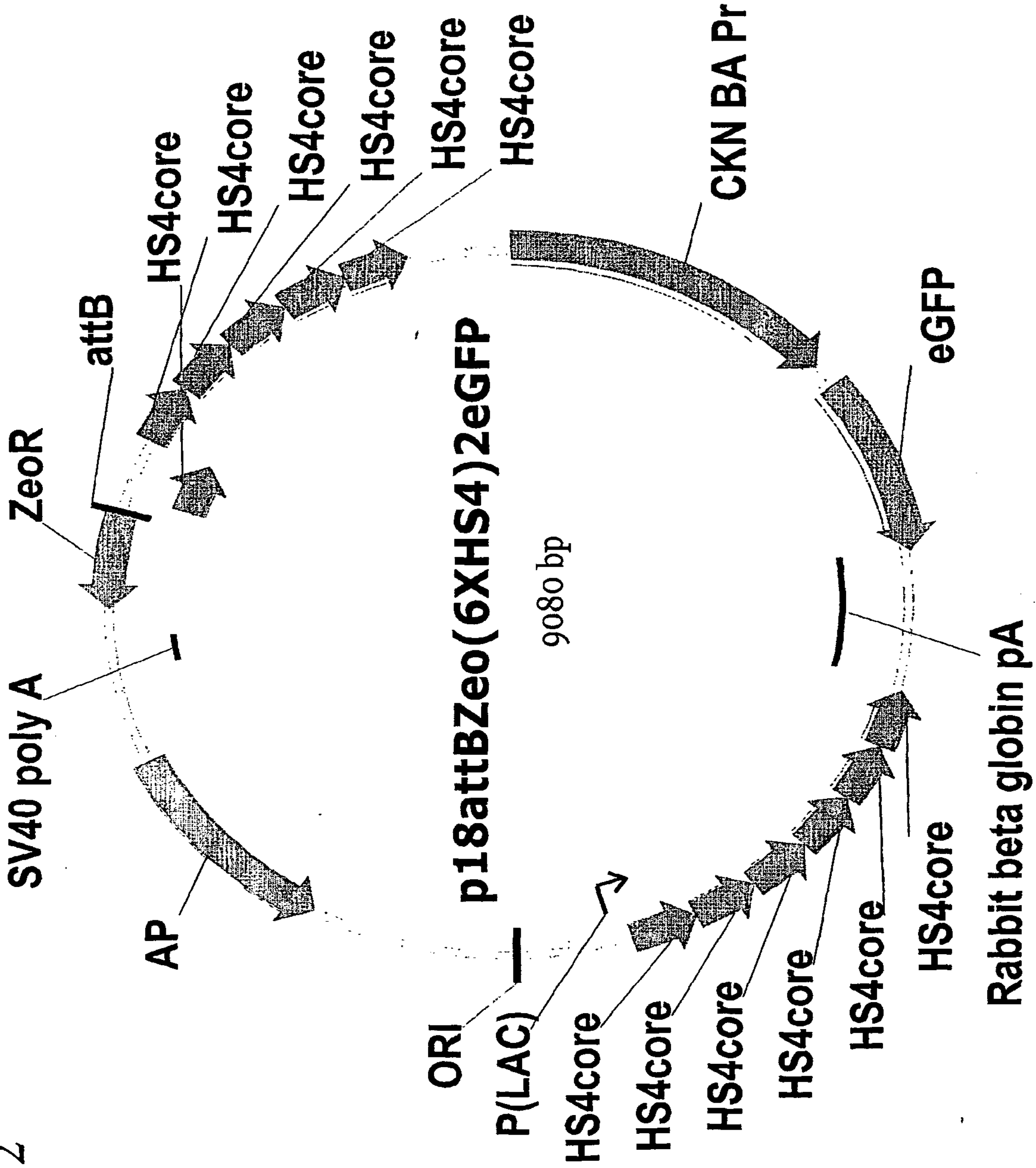
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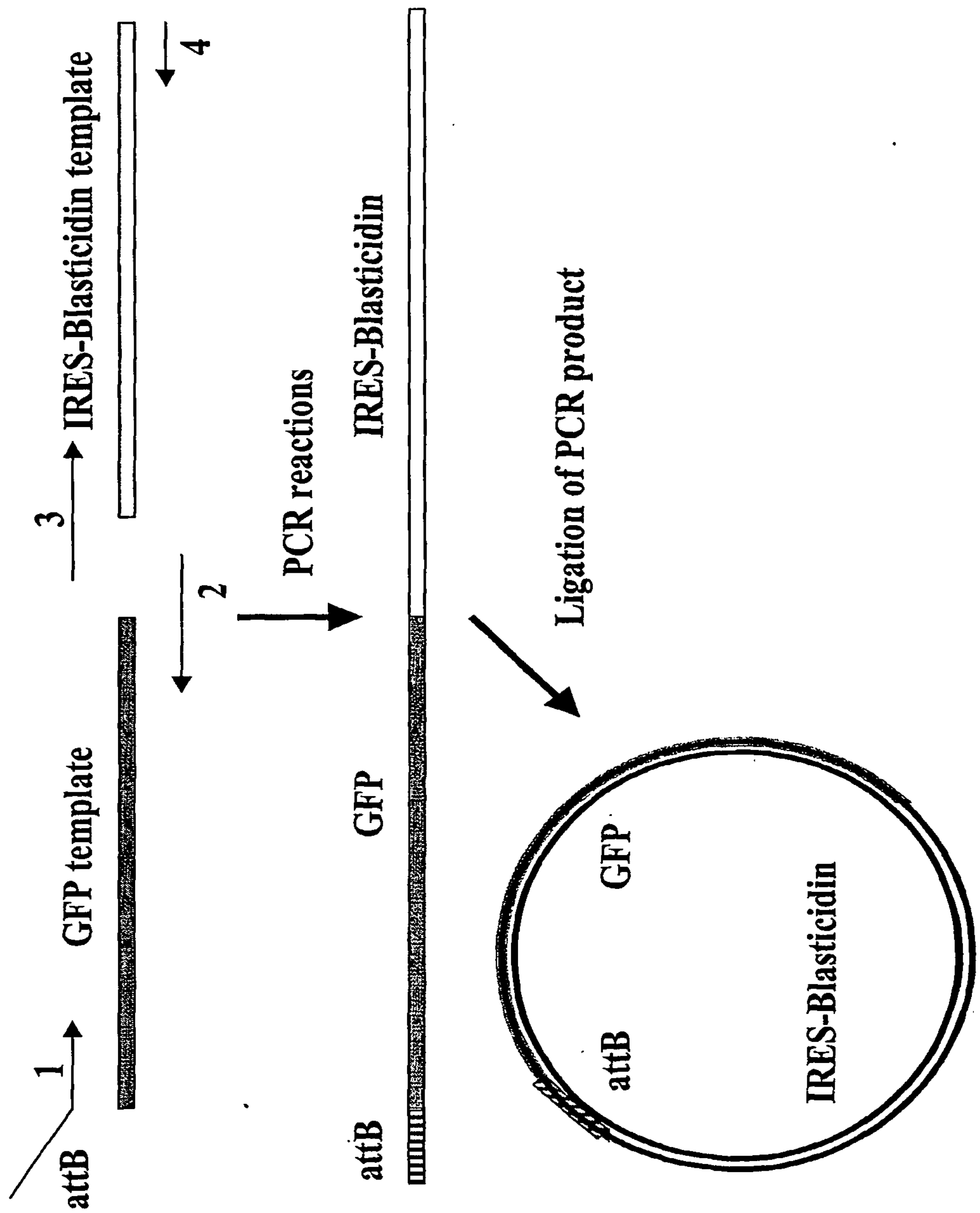
Fig. 12

Fig. 13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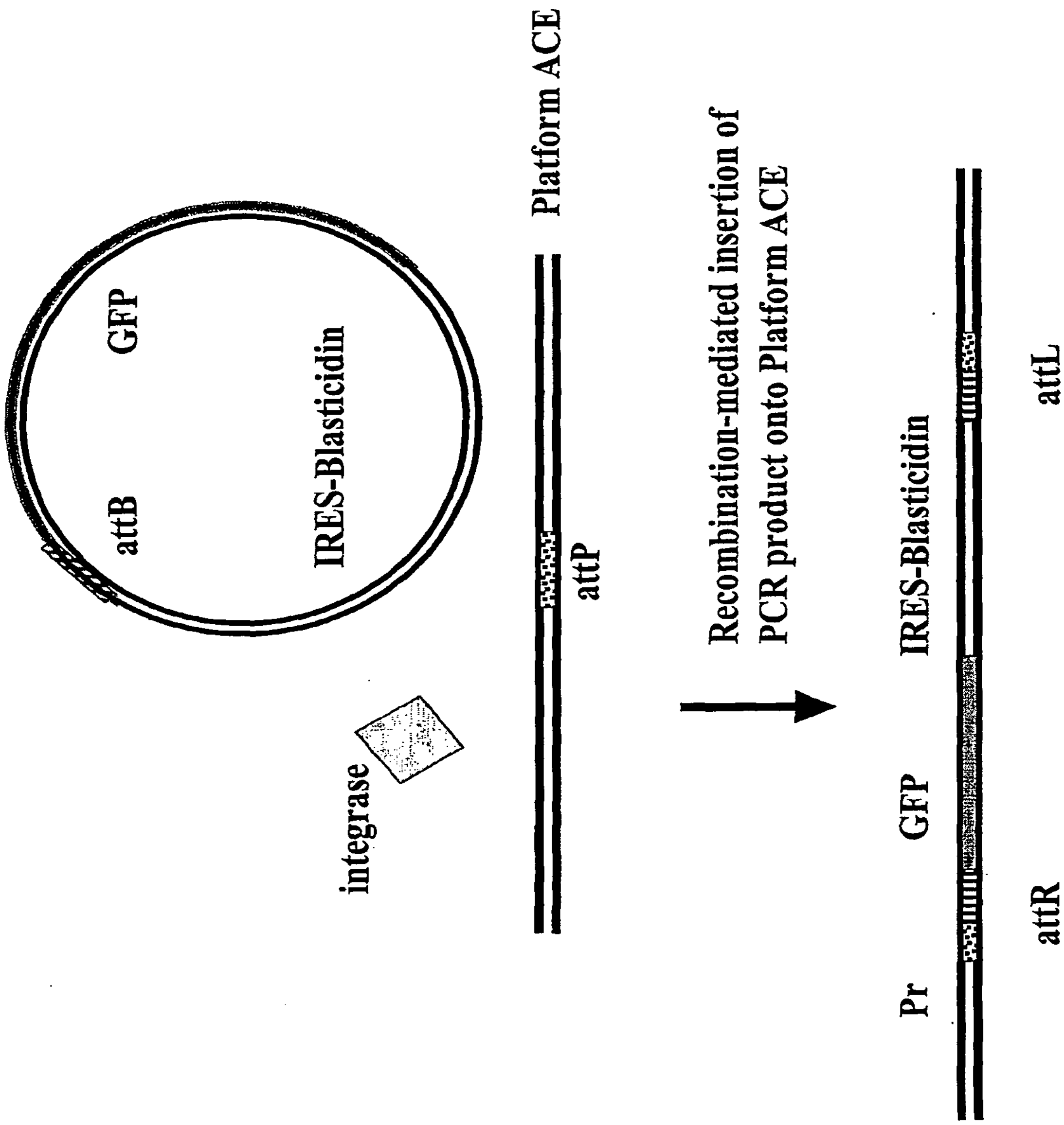
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Fig. 14

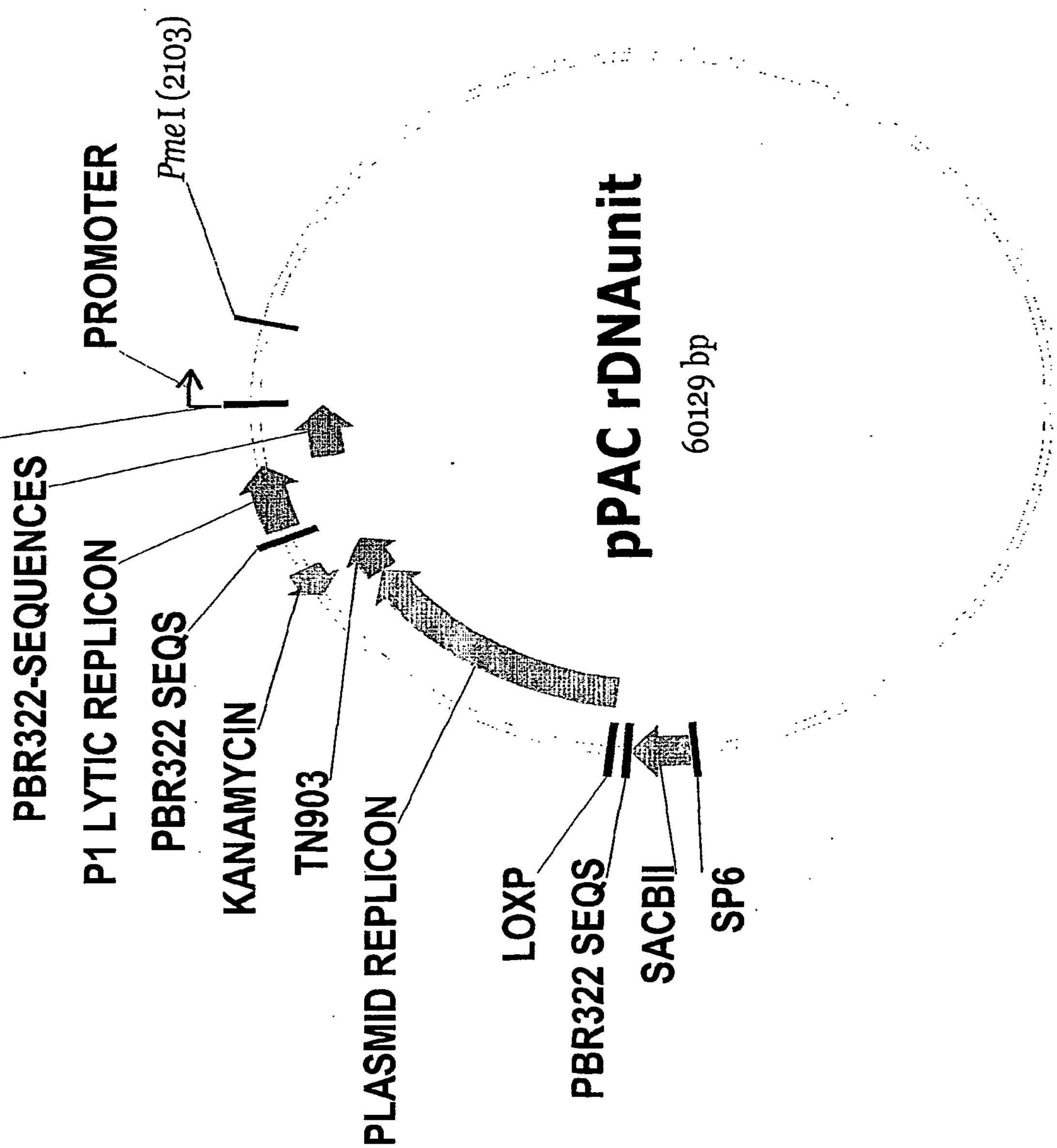
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Fig. 15

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<210> 27

<211> 5627
<212> DNA
<213> Artificial Sequence

<220>
<223> pMG plasmid from InvivoGen; IRES sequence modified
EMCV nucleotides 2736-3308

<400> 27
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<210> 28
<211> 553
<212> DNA
<213> Artificial Sequence

<220>
<223> pMG plasmid from InvivoGen: EMCV IRES sequence

<400> 28

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<210> 29
<211> 4692
<212> DNA
<213> Artificial Sequence

<220>
<223> pDSred1-N1 plasmid from Clontech

<400> 29

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<210> 30
<211> 4257
<212> DNA
<213> Artificial Sequence

<220>
<223> pPur plasmid from Clontech

<400> 30

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gtgtcacgct cgtcgtttgg tatggcttca ttcagctccg gttcccaacg atcaaggcga    2040
gttacatgat cccccatgtt gtgcaaaaaa gcggttagct ccttcggtcc tccgatcgtt    2100
gtcagaagta agttggccgc agtgttatca ctcatggtta tggcagcact gcataattct    2160
cttactgtca tgccatccgt aagatgcttt tctgtgactg gtgagtactc aaccaagtca    2220
ttctgagaat agtgtatgcg gcgaccgagt tgctcttgcc cggcgtcaat acgggataat    2280
accgcgccac atagcagaac tttaaaagtg ctcatcattg gaaaacgttc ttcggggcga    2340
aaactctcaa ggatcttacc gctgttgaga tccagttcga tgtaacccac tcgtgcaccc    2400
aactgatctt cagcatcttt tactttcacc agcgtttctg ggtgagcaaa aacaggaagg    2460
caaaatgccg caaaaaaggg aataagggcg acacggaaat gttgaatact catactcttc    2520
ctttttcaat attattgaag catttatcag ggttattgtc tcatgagcgg atacatattt    2580
gaatgtattt agaaaaataa acaaataggg gttccgcgca catttccccg aaaagtgcca    2640
cctgacgtct aagaaaccat tattatcatg acattaacct ataaaaatag gcgtatcacg    2700
aggccctttc gtc                                                         2713
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<210> 33
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> attP

<400> 33
cagctttttt atactaagtt g 21

<210> 34
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> attB

<400> 34
ctgctttttt atactaactt g 21

<210> 35
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> attL

<400> 35
ctgctttttt atactaagtt g 21

<210> 36
<211> 21
<212> DNA

<213> Artificial Sequence

<220>
<223> attR

<400> 36
cagctttttt atactaactt g 21

<210> 37
<211> 1071
<212> DNA
<213> Artificial Sequence

<220>
<223> Integrase E174R

<221> CDS
<222> (1)...(1071)
<223> Nucleotide sequence encoding Integrase E147R

<400> 37
atg gga aga agg cga agt cat gag cgc cgg gat tta ccc cct aac ctt 48
Met Gly Arg Arg Arg Ser His Glu Arg Arg Asp Leu Pro Pro Asn Leu
1 5 10 15

tat ata aga aac aat gga tat tac tgc tac agg gac cca agg acg ggt 96
Tyr Ile Arg Asn Asn Gly Tyr Tyr Cys Tyr Arg Asp Pro Arg Thr Gly
20 25 30

aaa gag ttt gga tta ggc aga gac agg cga atc gca atc act gaa gct 144
Lys Glu Phe Gly Leu Gly Arg Asp Arg Arg Ile Ala Ile Thr Glu Ala
35 40 45

ata cag gcc aac att gag tta ttt tca gga cac aaa cac aag cct ctg 192
Ile Gln Ala Asn Ile Glu Leu Phe Ser Gly His Lys His Lys Pro Leu
50 55 60

aca gcg aga atc aac agt gat aat tcc gtt acg tta cat tca tgg ctt 240
Thr Ala Arg Ile Asn Ser Asp Asn Ser Val Thr Leu His Ser Trp Leu
65 70 75 80

gat cgc tac gaa aaa atc ctg gcc agc aga gga atc aag cag aag aca 288
Asp Arg Tyr Glu Lys Ile Leu Ala Ser Arg Gly Ile Lys Gln Lys Thr
85 90 95

ctc ata aat tac atg agc aaa att aaa gca ata agg agg ggt ctg cct 336
Leu Ile Asn Tyr Met Ser Lys Ile Lys Ala Ile Arg Arg Gly Leu Pro
100 105 110

gat gct cca ctt gaa gac atc acc aca aaa gaa att gcg gca atg ctc 384
Asp Ala Pro Leu Glu Asp Ile Thr Thr Lys Glu Ile Ala Ala Met Leu
115 120 125

aat gga tac ata gac gag ggc aag gcg gcg tca gcc aag tta atc aga 432
Asn Gly Tyr Ile Asp Glu Gly Lys Ala Ala Ser Ala Lys Leu Ile Arg
130 135 140

tca aca ctg agc gat gca ttc cga gag gca ata gct gaa ggc cat ata 480
Ser Thr Leu Ser Asp Ala Phe Arg Glu Ala Ile Ala Glu Gly His Ile
145 150 155 160

aca aca aac cat gtc gct gcc act cgc gca gca aaa tct aga gta agg 528
Thr Thr Asn His Val Ala Ala Thr Arg Ala Ala Lys Ser Arg Val Arg
165 170 175

aga tca aga ctt acg gct gac gaa tac ctg aaa att tat caa gca gca 576
Arg Ser Arg Leu Thr Ala Asp Glu Tyr Leu Lys Ile Tyr Gln Ala Ala

```
            180                                     185                                     190

gaa tca tca cca tgt tgg ctc aga ctt gca atg gaa ctg gct gtt gtt      624
Glu Ser Ser Pro Cys Trp Leu Arg Leu Ala Met Glu Leu Ala Val Val
        195                 200                 205

acc ggg caa cga gtt ggt gat tta tgc gaa atg aag tgg tct gat atc      672
Thr Gly Gln Arg Val Gly Asp Leu Cys Glu Met Lys Trp Ser Asp Ile
    210                 215                 220

gta gat gga tat ctt tat gtc gag caa agc aaa aca ggc gta aaa att      720
Val Asp Gly Tyr Leu Tyr Val Glu Gln Ser Lys Thr Gly Val Lys Ile
225                 230                 235                 240

gcc atc cca aca gca ttg cat att gat gct ctc gga ata tca atg aag      768
Ala Ile Pro Thr Ala Leu His Ile Asp Ala Leu Gly Ile Ser Met Lys
                245                 250                 255

gaa aca ctt gat aaa tgc aaa gag att ctt ggc gga gaa acc ata att      816
Glu Thr Leu Asp Lys Cys Lys Glu Ile Leu Gly Gly Glu Thr Ile Ile
            260                 265                 270

gca tct act cgt cgc gaa ccg ctt tca tcc ggc aca gta tca agg tat      864
Ala Ser Thr Arg Arg Glu Pro Leu Ser Ser Gly Thr Val Ser Arg Tyr
        275                 280                 285

ttt atg cgc gca cga aaa gca tca ggt ctt tcc ttc gaa ggg gat ccg      912
Phe Met Arg Ala Arg Lys Ala Ser Gly Leu Ser Phe Glu Gly Asp Pro
    290                 295                 300

cct acc ttt cac gag ttg cgc agt ttg tct gca aga ctc tat gag aag      960
Pro Thr Phe His Glu Leu Arg Ser Leu Ser Ala Arg Leu Tyr Glu Lys
305                 310                 315                 320

cag ata agc gat aag ttt gct caa cat ctt ctc ggg cat aag tcg gac      1008
Gln Ile Ser Asp Lys Phe Ala Gln His Leu Leu Gly His Lys Ser Asp
                325                 330                 335

acc atg gca tca cag tat cgt gat gac aga ggc agg gag tgg gac aaa      1056
Thr Met Ala Ser Gln Tyr Arg Asp Asp Arg Gly Arg Glu Trp Asp Lys
            340                 345                 350

att gaa atc aaa taa                                                  1071
Ile Glu Ile Lys  *
        355
```

<210> 38
<211> 356
<212> PRT
<213> Artificial Sequence

<220>
<223> Integrase E147R

<400> 38

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Met Gly Arg Arg Arg Ser His Glu Arg Arg Asp Leu Pro Pro Asn Leu
 1               5                  10                  15
Tyr Ile Arg Asn Asn Gly Tyr Tyr Cys Tyr Arg Asp Pro Arg Thr Gly
            20                  25                  30
Lys Glu Phe Gly Leu Gly Arg Asp Arg Arg Ile Ala Ile Thr Glu Ala
        35                  40                  45
Ile Gln Ala Asn Ile Glu Leu Phe Ser Gly His Lys His Lys Pro Leu
    50                  55                  60
Thr Ala Arg Ile Asn Ser Asp Asn Ser Val Thr Leu His Ser Trp Leu
65                  70                  75                  80
Asp Arg Tyr Glu Lys Ile Leu Ala Ser Arg Gly Ile Lys Gln Lys Thr
```

```
                    85                        90                       95
Leu Ile Asn Tyr Met Ser Lys Ile Lys Ala Ile Arg Arg Gly Leu Pro
            100                      105                     110
Asp Ala Pro Leu Glu Asp Ile Thr Thr Lys Glu Ile Ala Ala Met Leu
        115                     120                     125
Asn Gly Tyr Ile Asp Glu Gly Lys Ala Ala Ser Ala Lys Leu Ile Arg
    130                     135                     140
Ser Thr Leu Ser Asp Ala Phe Arg Glu Ala Ile Ala Glu Gly His Ile
145                     150                     155                     160
Thr Thr Asn His Val Ala Ala Thr Arg Ala Ala Lys Ser Arg Val Arg
                165                     170                     175
Arg Ser Arg Leu Thr Ala Asp Glu Tyr Leu Lys Ile Tyr Gln Ala Ala
            180                     185                     190
Glu Ser Ser Pro Cys Trp Leu Arg Leu Ala Met Glu Leu Ala Val Val
        195                     200                     205
Thr Gly Gln Arg Val Gly Asp Leu Cys Glu Met Lys Trp Ser Asp Ile
    210                     215                     220
Val Asp Gly Tyr Leu Tyr Val Glu Gln Ser Lys Thr Gly Val Lys Ile
225                     230                     235                     240
Ala Ile Pro Thr Ala Leu His Ile Asp Ala Leu Gly Ile Ser Met Lys
                245                     250                     255
Glu Thr Leu Asp Lys Cys Lys Glu Ile Leu Gly Gly Glu Thr Ile Ile
            260                     265                     270
Ala Ser Thr Arg Arg Glu Pro Leu Ser Ser Gly Thr Val Ser Arg Tyr
        275                     280                     285
Phe Met Arg Ala Arg Lys Ala Ser Gly Leu Ser Phe Glu Gly Asp Pro
    290                     295                     300
Pro Thr Phe His Glu Leu Arg Ser Leu Ser Ala Arg Leu Tyr Glu Lys
305                     310                     315                     320
Gln Ile Ser Asp Lys Phe Ala Gln His Leu Leu Gly His Lys Ser Asp
                325                     330                     335
Thr Met Ala Ser Gln Tyr Arg Asp Asp Arg Gly Arg Glu Trp Asp Lys
            340                     345                     350
Ile Glu Ile Lys
        355
```

```
<210> 39
<211> 876
<212> DNA
<213> Discosoma species

<220>
<221> CDS
<222> (45)...(737)
<223> Nucleotide sequence encoding red flourescent
      protein (FP593)

<300>
<308> GenBank AF272711
<309> 2000-09-26

<400> 39
agtttcagcc agtgacaggg tgagctgcca ggtattctaa caag atg agt tgt tcc    56
                                                 Met Ser Cys Ser
                                                  1

aag aat gtg atc aag gag ttc atg agg ttc aag gtt cgt atg gaa gga    104
Lys Asn Val Ile Lys Glu Phe Met Arg Phe Lys Val Arg Met Glu Gly
 5                  10                  15                  20

acg gtc aat ggg cac gag ttt gaa ata aaa ggc gaa ggt gaa ggg agg    152
Thr Val Asn Gly His Glu Phe Glu Ile Lys Gly Glu Gly Glu Gly Arg
                25                  30                  35

cct tac gaa ggt cac tgt tcc gta aag ctt atg gta acc aag ggt gga    200
Pro Tyr Glu Gly His Cys Ser Val Lys Leu Met Val Thr Lys Gly Gly
```

```
                40                        45                        50

cct ttg cca ttt gct ttt gat att ttg tca cca caa ttt cag tat gga    248
Pro Leu Pro Phe Ala Phe Asp Ile Leu Ser Pro Gln Phe Gln Tyr Gly
        55                      60                      65

agc aag gta tat gtc aaa cac cct gcc gac ata cca gac tat aaa aag    296
Ser Lys Val Tyr Val Lys His Pro Ala Asp Ile Pro Asp Tyr Lys Lys
    70                      75                      80

ctg tca ttt cct gag gga ttt aaa tgg gaa agg gtc atg aac ttt gaa    344
Leu Ser Phe Pro Glu Gly Phe Lys Trp Glu Arg Val Met Asn Phe Glu
 85                      90                      95                 100

gac ggt ggc gtg gtt act gta tcc caa gat tcc agt ttg aaa gac ggc    392
Asp Gly Gly Val Val Thr Val Ser Gln Asp Ser Ser Leu Lys Asp Gly
                105                     110                     115

tgt ttc atc tac gag gtc aag ttc att ggg gtg aac ttt cct tct gat    440
Cys Phe Ile Tyr Glu Val Lys Phe Ile Gly Val Asn Phe Pro Ser Asp
            120                     125                     130

gga cct gtt atg cag agg agg aca cgg ggc tgg gaa gcc agc tct gag    488
Gly Pro Val Met Gln Arg Arg Thr Arg Gly Trp Glu Ala Ser Ser Glu
        135                     140                     145

cgt ttg tat cct cgt gat ggg gtg ctg aaa gga gac atc cat atg gct    536
Arg Leu Tyr Pro Arg Asp Gly Val Leu Lys Gly Asp Ile His Met Ala
    150                     155                     160

ctg agg ctg gaa gga ggc ggc cat tac ctc gtt gaa ttc aaa agt att    584
Leu Arg Leu Glu Gly Gly Gly His Tyr Leu Val Glu Phe Lys Ser Ile
165                     170                     175                 180

tac atg gta aag aag cct tca gtg cag ttg cca ggc tac tat tat gtt    632
Tyr Met Val Lys Lys Pro Ser Val Gln Leu Pro Gly Tyr Tyr Tyr Val
                185                     190                     195

gac tcc aaa ctg gat atg acg agc cac aac gaa gat tac aca gtc gtt    680
Asp Ser Lys Leu Asp Met Thr Ser His Asn Glu Asp Tyr Thr Val Val
            200                     205                     210

gag cag tat gaa aaa acc cag gga cgc cac cat ccg ttc att aag cct    728
Glu Gln Tyr Glu Lys Thr Gln Gly Arg His His Pro Phe Ile Lys Pro
        215                     220                     225

ctg cag tga actcggctca gtcatggatt agcggtaatg gccacaaaag            777
Leu Gln  *
    230

gcacgatgat cgtttttag gaatgcagcc aaaaattgaa ggttatgaca gtagaaatac 837
aagcaacagg ctttgcttat taaacatgta attgaaaac                       876
```

<210> 40
<211> 230
<212> PRT
<213> Discosoma species

<400> 40

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Met Ser Cys Ser Lys Asn Val Ile Lys Glu Phe Met Arg Phe Lys Val
 1               5                  10                  15
Arg Met Glu Gly Thr Val Asn Gly His Glu Phe Glu Ile Lys Gly Glu
            20                  25                  30
Gly Glu Gly Arg Pro Tyr Glu Gly His Cys Ser Val Lys Leu Met Val
        35                  40                  45
Thr Lys Gly Gly Pro Leu Pro Phe Ala Phe Asp Ile Leu Ser Pro Gln
    50                  55                  60
```

```
Phe Gln Tyr Gly Ser Lys Val Tyr Val Lys His Pro Ala Asp Ile Pro
65                  70                  75                  80
Asp Tyr Lys Lys Leu Ser Phe Pro Glu Gly Phe Lys Trp Glu Arg Val
                85                  90                  95
Met Asn Phe Glu Asp Gly Gly Val Val Thr Val Ser Gln Asp Ser Ser
            100                 105                 110
Leu Lys Asp Gly Cys Phe Ile Tyr Glu Val Lys Phe Ile Gly Val Asn
        115                 120                 125
Phe Pro Ser Asp Gly Pro Val Met Gln Arg Arg Thr Arg Gly Trp Glu
    130                 135                 140
Ala Ser Ser Glu Arg Leu Tyr Pro Arg Asp Gly Val Leu Lys Gly Asp
145                 150                 155                 160
Ile His Met Ala Leu Arg Leu Glu Gly Gly Gly His Tyr Leu Val Glu
                165                 170                 175
Phe Lys Ser Ile Tyr Met Val Lys Lys Pro Ser Val Gln Leu Pro Gly
            180                 185                 190
Tyr Tyr Tyr Val Asp Ser Lys Leu Asp Met Thr Ser His Asn Glu Asp
        195                 200                 205
Tyr Thr Val Val Glu Gln Tyr Glu Lys Thr Gln Gly Arg His His Pro
    210                 215                 220
Phe Ile Lys Pro Leu Gln
225                 230
```

<210> 41
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> m-att;

<221> misc_difference
<222> 18
<223> n is a or g or c or t/u

<400> 41
rkycwgcttt yktrtacnaa stsgb 25

<210> 42
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> m-attB;

<221> misc_difference
<222> 18
<223> n is a or g or c or t/u

<400> 42
agccwgcttt yktrtacnaa ctsgb 25

<210> 43
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> m-attR

<221> misc_difference
<222> 18
<223> n is a or g or c or t/u

<400> 43
gttcagcttt cktrtacnaa ctsgb 25

<210> 44
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> m-attL

<221> misc_difference
<222> 18
<223> n is a or g or c or t/u

<400> 44
agccwgcttt cktrtacnaa gtsgb 25

<210> 45
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> m-attP1

<221> misc_difference
<222> 18
<223> n is a or g or c or t/u

<400> 45
gttcagcttt yktrtacnaa gtsgb 25

<210> 46
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attB1

<400> 46
agcctgcttt tttgtacaaa cttgt 25

<210> 47
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attB2

<400> 47
agcctgcttt cttgtacaaa cttgt 25

<210> 48
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attB3

<400> 48
acccagcttt cttgtacaaa cttgt 25

<210> 49

<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attR1

<400> 49
gttcagcttt tttgtacaaa cttgt 25

<210> 50
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attR2

<400> 50
gttcagcttt cttgtacaaa cttgt 25

<210> 51
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attR3

<400> 51
gttcagcttt cttgtacaaa gttgg 25

<210> 52
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attL1

<400> 52
agcctgcttt tttgtacaaa gttgg 25

<210> 53
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attL2

<400> 53
agcctgcttt cttgtacaaa gttgg 25

<210> 54
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attL3

<400> 54
acccagcttt cttgtacaaa gttgg 25

<210> 55
<211> 25

<212> DNA
<213> Artificial Sequence

<220>
<223> attP1

<400> 55
gttcagcttt tttgtacaaa gttgg 25

<210> 56
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attP2,P3

<400> 56
gttcagcttt cttgtacaaa gttgg 25

<210> 57
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Lox P site

<400> 57
ataacttcgt ataatgtatg ctatacgaag ttat 34

<210> 58
<211> 1032
<212> DNA
<213> Escherichia coli

<220>
<221> CDS
<222> (1)...(1032)
<223> nucleotide sequence encoding Cre recombinase

<400> 58
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atg tcc aat tta ctg acc gta cac caa aat ttg cct gca tta ccg gtc       48
Met Ser Asn Leu Leu Thr Val His Gln Asn Leu Pro Ala Leu Pro Val
 1               5                  10                  15

gat gca acg agt gat gag gtt cgc aag aac ctg atg gac atg ttc agg       96
Asp Ala Thr Ser Asp Glu Val Arg Lys Asn Leu Met Asp Met Phe Arg
            20                  25                  30

gat cgc cag gcg ttt tct gag cat acc tgg aaa atg ctt ctg tcc gtt      144
Asp Arg Gln Ala Phe Ser Glu His Thr Trp Lys Met Leu Leu Ser Val
        35                  40                  45

tgc cgg tcg tgg gcg gca tgg tgc aag ttg aat aac cgg aaa tgg ttt      192
Cys Arg Ser Trp Ala Ala Trp Cys Lys Leu Asn Asn Arg Lys Trp Phe
    50                  55                  60

ccc gca gaa cct gaa gat gtt cgc gat tat ctt cta tat ctt cag gcg      240
Pro Ala Glu Pro Glu Asp Val Arg Asp Tyr Leu Leu Tyr Leu Gln Ala
65                  70                  75                  80

cgc ggt ctg gca gta aaa act atc cag caa cat ttg ggc cag cta aac      288
Arg Gly Leu Ala Val Lys Thr Ile Gln Gln His Leu Gly Gln Leu Asn
                85                  90                  95

atg ctt cat cgt cgg tcc ggg ctg cca cga cca agt gac agc aat gct      336
```

Met Leu His Arg Arg Ser Gly Leu Pro Arg Pro Ser Asp Ser Asn Ala
100 105 110

gtt tca ctg gtt atg cgg cgg atc cga aaa gaa aac gtt gat gcc ggt 384
Val Ser Leu Val Met Arg Arg Ile Arg Lys Glu Asn Val Asp Ala Gly
115 120 125

gaa cgt gca aaa cag gct cta gcg ttc gaa cgc act gat ttc gac cag 432
Glu Arg Ala Lys Gln Ala Leu Ala Phe Glu Arg Thr Asp Phe Asp Gln
130 135 140

gtt cgt tca ctc atg gaa aat agc gat cgc tgc cag gat ata cgt aat 480
Val Arg Ser Leu Met Glu Asn Ser Asp Arg Cys Gln Asp Ile Arg Asn
145 150 155 160

ctg gca ttt ctg ggg att gct tat aac acc ctg tta cgt ata gcc gaa 528
Leu Ala Phe Leu Gly Ile Ala Tyr Asn Thr Leu Leu Arg Ile Ala Glu
165 170 175

att gcc agg atc agg gtt aaa gat atc tca cgt act gac ggt ggg aga 576
Ile Ala Arg Ile Arg Val Lys Asp Ile Ser Arg Thr Asp Gly Gly Arg
180 185 190

atg tta atc cat att ggc aga acg aaa acg ctg gtt agc acc gca ggt 624
Met Leu Ile His Ile Gly Arg Thr Lys Thr Leu Val Ser Thr Ala Gly
195 200 205

gta gag aag gca ctt agc ctg ggg gta act aaa ctg gtc gag cga tgg 672
Val Glu Lys Ala Leu Ser Leu Gly Val Thr Lys Leu Val Glu Arg Trp
210 215 220

att tcc gtc tct ggt gta gct gat gat ccg aat aac tac ctg ttt tgc 720
Ile Ser Val Ser Gly Val Ala Asp Asp Pro Asn Asn Tyr Leu Phe Cys
225 230 235 240

cgg gtc aga aaa aat ggt gtt gcc gcg cca tct gcc acc agc cag cta 768
Arg Val Arg Lys Asn Gly Val Ala Ala Pro Ser Ala Thr Ser Gln Leu
245 250 255

tca act cgc gcc ctg gaa ggg att ttt gaa gca act cat cga ttg att 816
Ser Thr Arg Ala Leu Glu Gly Ile Phe Glu Ala Thr His Arg Leu Ile
260 265 270

tac ggc gct aag gat gac tct ggt cag aga tac ctg gcc tgg tct gga 864
Tyr Gly Ala Lys Asp Asp Ser Gly Gln Arg Tyr Leu Ala Trp Ser Gly
275 280 285

cac agt gcc cgt gtc gga gcc gcg cga gat atg gcc cgc gct gga gtt 912
His Ser Ala Arg Val Gly Ala Ala Arg Asp Met Ala Arg Ala Gly Val
290 295 300

tca ata ccg gag atc atg caa gct ggt ggc tgg acc aat gta aat att 960
Ser Ile Pro Glu Ile Met Gln Ala Gly Gly Trp Thr Asn Val Asn Ile
305 310 315 320

gtc atg aac tat atc cgt aac ctg gat agt gaa aca ggg gca atg gtg 1008
Val Met Asn Tyr Ile Arg Asn Leu Asp Ser Glu Thr Gly Ala Met Val
325 330 335

cgc ctg ctg gaa gat ggc gat tag 1032
Arg Leu Leu Glu Asp Gly Asp *
340

<210> 59
<211> 343
<212> PRT

<213> Escherichia coli

<400> 59
Met Ser Asn Leu Leu Thr Val His Gln Asn Leu Pro Ala Leu Pro Val
1   5   10   15
Asp Ala Thr Ser Asp Glu Val Arg Lys Asn Leu Met Asp Met Phe Arg
            20   25   30
Asp Arg Gln Ala Phe Ser Glu His Thr Trp Lys Met Leu Leu Ser Val
        35   40   45
Cys Arg Ser Trp Ala Ala Trp Cys Lys Leu Asn Asn Arg Lys Trp Phe
    50   55   60
Pro Ala Glu Pro Glu Asp Val Arg Asp Tyr Leu Leu Tyr Leu Gln Ala
65   70   75   80
Arg Gly Leu Ala Val Lys Thr Ile Gln Gln His Leu Gly Gln Leu Asn
                85   90   95
Met Leu His Arg Arg Ser Gly Leu Pro Arg Pro Ser Asp Ser Asn Ala
            100   105   110
Val Ser Leu Val Met Arg Arg Ile Arg Lys Glu Asn Val Asp Ala Gly
        115   120   125
Glu Arg Ala Lys Gln Ala Leu Ala Phe Glu Arg Thr Asp Phe Asp Gln
    130   135   140
Val Arg Ser Leu Met Glu Asn Ser Asp Arg Cys Gln Asp Ile Arg Asn
145   150   155   160
Leu Ala Phe Leu Gly Ile Ala Tyr Asn Thr Leu Leu Arg Ile Ala Glu
                165   170   175
Ile Ala Arg Ile Arg Val Lys Asp Ile Ser Arg Thr Asp Gly Gly Arg
            180   185   190
Met Leu Ile His Ile Gly Arg Thr Lys Thr Leu Val Ser Thr Ala Gly
        195   200   205
Val Glu Lys Ala Leu Ser Leu Gly Val Thr Lys Leu Val Glu Arg Trp
    210   215   220
Ile Ser Val Ser Gly Val Ala Asp Asp Pro Asn Asn Tyr Leu Phe Cys
225   230   235   240
Arg Val Arg Lys Asn Gly Val Ala Ala Pro Ser Ala Thr Ser Gln Leu
                245   250   255
Ser Thr Arg Ala Leu Glu Gly Ile Phe Glu Ala Thr His Arg Leu Ile
            260   265   270
Tyr Gly Ala Lys Asp Asp Ser Gly Gln Arg Tyr Leu Ala Trp Ser Gly
        275   280   285
His Ser Ala Arg Val Gly Ala Ala Arg Asp Met Ala Arg Ala Gly Val
    290   295   300
Ser Ile Pro Glu Ile Met Gln Ala Gly Gly Trp Thr Asn Val Asn Ile
305   310   315   320
Val Met Asn Tyr Ile Arg Asn Leu Asp Ser Glu Thr Gly Ala Met Val
                325   330   335
Arg Leu Leu Glu Asp Gly Asp
            340

<210> 60
<211> 1272
<212> DNA
<213> Saccharomyces cerevisiae

<220>
<221> CDS
<222> (1)...(1272)
<223> nucleotide sequence encoding Flip recombinase

<400> 60
atg cca caa ttt ggt ata tta tgt aaa aca cca cct aag gtg ctt gtt   48
Met Pro Gln Phe Gly Ile Leu Cys Lys Thr Pro Pro Lys Val Leu Val
1   5   10   15

cgt cag ttt gtg gaa agg ttt gaa aga cct tca ggt gag aaa ata gca   96
Arg Gln Phe Val Glu Arg Phe Glu Arg Pro Ser Gly Glu Lys Ile Ala
            20   25   30

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tta tgt gct gct gaa cta acc tat tta tgt tgg atg att aca cat aac      144
Leu Cys Ala Ala Glu Leu Thr Tyr Leu Cys Trp Met Ile Thr His Asn
         35                  40                  45

gga aca gca atc aag aga gcc aca ttc atg agc tat aat act atc ata      192
Gly Thr Ala Ile Lys Arg Ala Thr Phe Met Ser Tyr Asn Thr Ile Ile
     50                  55                  60

agc aat tcg ctg agt ttc gat att gtc aat aaa tca ctc cag ttt aaa      240
Ser Asn Ser Leu Ser Phe Asp Ile Val Asn Lys Ser Leu Gln Phe Lys
 65                  70                  75                  80

tac aag acg caa aaa gca aca att ctg gaa gcc tca tta aag aaa ttg      288
Tyr Lys Thr Gln Lys Ala Thr Ile Leu Glu Ala Ser Leu Lys Lys Leu
                 85                  90                  95

att cct gct tgg gaa ttt aca att att cct tac tat gga caa aaa cat      336
Ile Pro Ala Trp Glu Phe Thr Ile Ile Pro Tyr Tyr Gly Gln Lys His
            100                 105                 110

caa tct gat atc act gat att gta agt agt ttg caa tta cag ttc gaa      384
Gln Ser Asp Ile Thr Asp Ile Val Ser Ser Leu Gln Leu Gln Phe Glu
        115                 120                 125

tca tcg gaa gaa gca gat aag gga aat agc cac agt aaa aaa atg ctt      432
Ser Ser Glu Glu Ala Asp Lys Gly Asn Ser His Ser Lys Lys Met Leu
    130                 135                 140

aaa gca ctt cta agt gag ggt gaa agc atc tgg gag atc act gag aaa      480
Lys Ala Leu Leu Ser Glu Gly Glu Ser Ile Trp Glu Ile Thr Glu Lys
145                 150                 155                 160

ata cta aat tcg ttt gag tat act tcg aga ttt aca aaa aca aaa act      528
Ile Leu Asn Ser Phe Glu Tyr Thr Ser Arg Phe Thr Lys Thr Lys Thr
                165                 170                 175

tta tac caa ttc ctc ttc cta gct act ttc atc aat tgt gga aga ttc      576
Leu Tyr Gln Phe Leu Phe Leu Ala Thr Phe Ile Asn Cys Gly Arg Phe
            180                 185                 190

agc gat att aag aac gtt gat ccg aaa tca ttt aaa tta gtc caa aat      624
Ser Asp Ile Lys Asn Val Asp Pro Lys Ser Phe Lys Leu Val Gln Asn
        195                 200                 205

aag tat ctg gga gta ata atc cag tgt tta gtg aca gag aca aag aca      672
Lys Tyr Leu Gly Val Ile Ile Gln Cys Leu Val Thr Glu Thr Lys Thr
    210                 215                 220

agc gtt agt agg cac ata tac ttc ttt agc gca agg ggt agg atc gat      720
Ser Val Ser Arg His Ile Tyr Phe Phe Ser Ala Arg Gly Arg Ile Asp
225                 230                 235                 240

cca ctt gta tat ttg gat gaa ttt ttg agg aat tct gaa cca gtc cta      768
Pro Leu Val Tyr Leu Asp Glu Phe Leu Arg Asn Ser Glu Pro Val Leu
                245                 250                 255

aaa cga gta aat agg acc ggc aat tct tca agc aat aaa cag gaa tac      816
Lys Arg Val Asn Arg Thr Gly Asn Ser Ser Ser Asn Lys Gln Glu Tyr
            260                 265                 270

caa tta tta aaa gat aac tta gtc aga tcg tac aat aaa gct ttg aag      864
Gln Leu Leu Lys Asp Asn Leu Val Arg Ser Tyr Asn Lys Ala Leu Lys
        275                 280                 285

aaa aat gcg cct tat tca atc ttt gct ata aaa aat ggc cca aaa tct      912
Lys Asn Ala Pro Tyr Ser Ile Phe Ala Ile Lys Asn Gly Pro Lys Ser
    290                 295                 300
```

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cac att gga aga cat ttg atg acc tca ttt ctt tca atg aag ggc cta      960
His Ile Gly Arg His Leu Met Thr Ser Phe Leu Ser Met Lys Gly Leu
305                 310                 315                 320

acg gag ttg act aat gtt gtg gga aat tgg agc gat aag cgt gct tct     1008
Thr Glu Leu Thr Asn Val Val Gly Asn Trp Ser Asp Lys Arg Ala Ser
                325                 330                 335

gcc gtg gcc agg aca acg tat act cat cag ata aca gca ata cct gat     1056
Ala Val Ala Arg Thr Thr Tyr Thr His Gln Ile Thr Ala Ile Pro Asp
            340                 345                 350

cac tac ttc gca cta gtt tct cgg tac tat gca tat gat cca ata tca     1104
His Tyr Phe Ala Leu Val Ser Arg Tyr Tyr Ala Tyr Asp Pro Ile Ser
        355                 360                 365

aag gaa atg ata gca ttg aag gat gag act aat cca att gag gag tgg     1152
Lys Glu Met Ile Ala Leu Lys Asp Glu Thr Asn Pro Ile Glu Glu Trp
    370                 375                 380

cag cat ata gaa cag cta aag ggt agt gct gaa gga agc ata cga tac     1200
Gln His Ile Glu Gln Leu Lys Gly Ser Ala Glu Gly Ser Ile Arg Tyr
385                 390                 395                 400

ccc gca tgg aat ggg ata ata tca cag gag gta cta gac tac ctt tca     1248
Pro Ala Trp Asn Gly Ile Ile Ser Gln Glu Val Leu Asp Tyr Leu Ser
                405                 410                 415

tcc tac ata aat aga cgc ata taa                                     1272
Ser Tyr Ile Asn Arg Arg Ile  *
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<210> 61
<211> 422
<212> PRT
<213> Saccharomyces cerevisiae

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Pro Gln Phe Gly Ile Leu Cys Lys Thr Pro Pro Lys Val Leu Val Arg
1               5                   10                  15
Gln Phe Val Glu Arg Phe Glu Arg Pro Ser Gly Glu Lys Ile Ala Leu
            20                  25                  30
Cys Ala Ala Glu Leu Thr Tyr Leu Cys Trp Met Ile Thr His Asn Gly
        35                  40                  45
Thr Ala Ile Lys Arg Ala Thr Phe Met Ser Tyr Asn Thr Ile Ile Ser
    50                  55                  60
Asn Ser Leu Ser Phe Asp Ile Val Asn Lys Ser Leu Gln Phe Lys Tyr
65                  70                  75                  80
Lys Thr Gln Lys Ala Thr Ile Leu Glu Ala Ser Leu Lys Lys Leu Ile
                85                  90                  95
Pro Ala Trp Glu Phe Thr Ile Ile Pro Tyr Tyr Gly Gln Lys His Gln
            100                 105                 110
Ser Asp Ile Thr Asp Ile Val Ser Ser Leu Gln Leu Gln Phe Glu Ser
        115                 120                 125
Ser Glu Glu Ala Asp Lys Gly Asn Ser His Ser Lys Lys Met Leu Lys
    130                 135                 140
Ala Leu Leu Ser Glu Gly Glu Ser Ile Trp Glu Ile Thr Glu Lys Ile
145                 150                 155                 160
Leu Asn Ser Phe Glu Tyr Thr Ser Arg Phe Thr Lys Thr Lys Thr Leu
                165                 170                 175
Tyr Gln Phe Leu Phe Leu Ala Thr Phe Ile Asn Cys Gly Arg Phe Ser
            180                 185                 190
Asp Ile Lys Asn Val Asp Pro Lys Ser Phe Lys Leu Val Gln Asn Lys
        195                 200                 205
Tyr Leu Gly Val Ile Ile Gln Cys Leu Val Thr Glu Thr Lys Thr Ser
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210 215 220
Val Ser Arg His Ile Tyr Phe Phe Ser Ala Arg Gly Arg Ile Asp Pro
225 230 235 240
Leu Val Tyr Leu Asp Glu Phe Leu Arg Asn Ser Glu Pro Val Leu Lys
245 250 255
Arg Val Asn Arg Thr Gly Asn Ser Ser Ser Asn Lys Gln Glu Tyr Gln
260 265 270
Leu Leu Lys Asp Asn Leu Val Arg Ser Tyr Asn Lys Ala Leu Lys Lys
275 280 285
Asn Ala Pro Tyr Ser Ile Phe Ala Ile Lys Asn Gly Pro Lys Ser His
290 295 300
Ile Gly Arg His Leu Met Thr Ser Phe Leu Ser Met Lys Gly Leu Thr
305 310 315 320
Glu Leu Thr Asn Val Val Gly Asn Trp Ser Asp Lys Arg Ala Ser Ala
325 330 335
Val Ala Arg Thr Thr Tyr Thr His Gln Ile Thr Ala Ile Pro Asp His
340 345 350
Tyr Phe Ala Leu Val Ser Arg Tyr Tyr Ala Tyr Asp Pro Ile Ser Lys
355 360 365
Glu Met Ile Ala Leu Lys Asp Glu Thr Asn Pro Ile Glu Glu Trp Gln
370 375 380
His Ile Glu Gln Leu Lys Gly Ser Ala Glu Gly Ser Ile Arg Tyr Pro
385 390 395 400
Ala Trp Asn Gly Ile Ile Ser Gln Glu Val Leu Asp Tyr Leu Ser Ser
405 410 415
Tyr Ile Asn Arg Arg Ile
420

<210> 62
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> IR2

<400> 62
gaagttccta ttccgaagtt cctattctct agaaagtata ggaacttc 48

<210> 63
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> IR1

<400> 63
gaagttccta tactttctag agaataggaa cttcggaata ggaacttc 48

<210> 64
<211> 66
<212> DNA
<213> Bacteriophage mu

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<221> CDS
<222> (1)...(66)
<223> nucleotide sequence encoding GIN recombinase

<400> 64
tca act ctg tat aaa aaa cac ccc gcg aaa cga gcg cat ata gaa aac 48
Ser Thr Leu Tyr Lys Lys His Pro Ala Lys Arg Ala His Ile Glu Asn
1 5 10 15

gac gat cga atc aat taa 66
Asp Asp Arg Ile Asn *

20

<210> 65
<211> 21
<212> PRT
<213> bacteriophage mu

<400> 65
Ser Thr Leu Tyr Lys Lys His Pro Ala Lys Arg Ala His Ile Glu Asn
1 5 10 15
Asp Asp Arg Ile Asn
20

<210> 66
<211> 69
<212> DNA
<213> Bacteriophage mu

<220>
<221> CDS
<222> (1)...(69)
<223> nucleotide sequence encoding Gin recombinase

<400> 66
tat aaa aaa cat ccc gcg aaa cga acg cat ata gaa aac gac gat cga 48
Tyr Lys Lys His Pro Ala Lys Arg Thr His Ile Glu Asn Asp Asp Arg
1 5 10 15

atc aat caa atc gat cgg taa 69
Ile Asn Gln Ile Asp Arg *
20

<210> 67
<211> 22
<212> PRT
<213> bacteriophage mu

<220>
<223> Gin recombinase of bacteriophage mu

<400> 67
Tyr Lys Lys His Pro Ala Lys Arg Thr His Ile Glu Asn Asp Asp Arg
1 5 10 15
Ile Asn Gln Ile Asp Arg
20

<210> 68
<211> 555
<212> DNA
<213> Escherichia coli

<220>
<221> CDS
<222> (1)...(555)
<223> nucleotide sequence encoding PIN recombinase

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atg ctt att ggc tat gta cgc gta tca aca aat gac cag aac aca gat 48
Met Leu Ile Gly Tyr Val Arg Val Ser Thr Asn Asp Gln Asn Thr Asp
1 5 10 15

cta caa cgt aat gcg ctg aac tgt gca gga tgc gag ctg att ttt gaa 96
Leu Gln Arg Asn Ala Leu Asn Cys Ala Gly Cys Glu Leu Ile Phe Glu
20 25 30

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gac aag ata agc ggc aca aag tcc gaa agg ccg gga ctg aaa aaa ctg       144
Asp Lys Ile Ser Gly Thr Lys Ser Glu Arg Pro Gly Leu Lys Lys Leu
        35                  40                  45

ctc agg aca tta tcg gca ggt gac act ctg gtt gtc tgg aag ctg gat       192
Leu Arg Thr Leu Ser Ala Gly Asp Thr Leu Val Val Trp Lys Leu Asp
    50                  55                  60

cgg ctg ggg cgt agt atg cgg cat ctt gtc gtg ctg gtg gag gag ttg       240
Arg Leu Gly Arg Ser Met Arg His Leu Val Val Leu Val Glu Glu Leu
65                  70                  75                  80

cgc gaa cga ggc atc aac ttt cgt agt ctg acg gat tca att gat acc       288
Arg Glu Arg Gly Ile Asn Phe Arg Ser Leu Thr Asp Ser Ile Asp Thr
                85                  90                  95

agc aca cca atg gga cgc ttt ttc ttt cat gtg atg ggt gcc ctg gct       336
Ser Thr Pro Met Gly Arg Phe Phe Phe His Val Met Gly Ala Leu Ala
            100                 105                 110

gaa atg gag cgt gaa ctg att gtt gaa cga aca aaa gct gga ctg gaa       384
Glu Met Glu Arg Glu Leu Ile Val Glu Arg Thr Lys Ala Gly Leu Glu
        115                 120                 125

act gct cgt gca cag gga cga att ggt gga cgt cgt ccc aaa ctt aca       432
Thr Ala Arg Ala Gln Gly Arg Ile Gly Gly Arg Arg Pro Lys Leu Thr
    130                 135                 140

cca gaa caa tgg gca caa gct gga cga tta att gca gca gga act cct       480
Pro Glu Gln Trp Ala Gln Ala Gly Arg Leu Ile Ala Ala Gly Thr Pro
145                 150                 155                 160

cgc cag aag gtg gcg att atc tat gat gtt ggt gtg tca act ttg tat       528
Arg Gln Lys Val Ala Ile Ile Tyr Asp Val Gly Val Ser Thr Leu Tyr
                165                 170                 175

aag agg ttt cct gca ggg gat aaa taa                                   555
Lys Arg Phe Pro Ala Gly Asp Lys  *
            180


<210> 69
<211> 184
<212> PRT
<213> Escherichia coli

<400> 69
Met Leu Ile Gly Tyr Val Arg Val Ser Thr Asn Asp Gln Asn Thr Asp
1               5                   10                  15
Leu Gln Arg Asn Ala Leu Asn Cys Ala Gly Cys Glu Leu Ile Phe Glu
            20                  25                  30
Asp Lys Ile Ser Gly Thr Lys Ser Glu Arg Pro Gly Leu Lys Lys Leu
        35                  40                  45
Leu Arg Thr Leu Ser Ala Gly Asp Thr Leu Val Val Trp Lys Leu Asp
    50                  55                  60
Arg Leu Gly Arg Ser Met Arg His Leu Val Val Leu Val Glu Glu Leu
65                  70                  75                  80
Arg Glu Arg Gly Ile Asn Phe Arg Ser Leu Thr Asp Ser Ile Asp Thr
                85                  90                  95
Ser Thr Pro Met Gly Arg Phe Phe Phe His Val Met Gly Ala Leu Ala
            100                 105                 110
Glu Met Glu Arg Glu Leu Ile Val Glu Arg Thr Lys Ala Gly Leu Glu
        115                 120                 125
Thr Ala Arg Ala Gln Gly Arg Ile Gly Gly Arg Arg Pro Lys Leu Thr
    130                 135                 140
Pro Glu Gln Trp Ala Gln Ala Gly Arg Leu Ile Ala Ala Gly Thr Pro
145                 150                 155                 160
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Arg Gln Lys Val Ala Ile Ile Tyr Asp Val Gly Val Ser Thr Leu Tyr
            165                 170                 175
Lys Arg Phe Pro Ala Gly Asp Lys
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<211> 4778
<212> DNA
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<220>
<223> pcx plasmid

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<210> 71
<211> 5510
<212> DNA
<213> Artificial Sequence

<220>
<223> pCXeGFP plasmid

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att ggg gcc aac tcc tac cgt acc tcg cat tac cct tac gct gaa gag     1008
Ile Gly Ala Asn Ser Tyr Arg Thr Ser His Tyr Pro Tyr Ala Glu Glu
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atg ctc gac tgg gca gat gaa cat ggc atc gtg gtg att gat gaa act     1056
Met Leu Asp Trp Ala Asp Glu His Gly Ile Val Val Ile Asp Glu Thr
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Ala Ala Val Gly Phe Asn Leu Ser Leu Gly Ile Gly Phe Glu Ala Gly
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aac aag ccg aaa gaa ctg tac agc gaa gag gca gtc aac ggg gaa act     1152
Asn Lys Pro Lys Glu Leu Tyr Ser Glu Glu Ala Val Asn Gly Glu Thr
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Gln Gln Ala His Leu Gln Ala Ile Lys Glu Leu Ile Ala Arg Asp Lys
385                 390                 395                 400

aac cac cca agc gtg gtg atg tgg agt att gcc aac gaa ccg gat acc     1248
Asn His Pro Ser Val Val Met Trp Ser Ile Ala Asn Glu Pro Asp Thr
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cgt ccg caa ggt gca cgg gaa tat ttc gcg cca ctg gcg gaa gca acg     1296
Arg Pro Gln Gly Ala Arg Glu Tyr Phe Ala Pro Leu Ala Glu Ala Thr
            420                 425                 430

cgt aaa ctc gac ccg acg cgt ccg atc acc tgc gtc aat gta atg ttc     1344
Arg Lys Leu Asp Pro Thr Arg Pro Ile Thr Cys Val Asn Val Met Phe
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Cys Asp Ala His Thr Asp Thr Ile Ser Asp Leu Phe Asp Val Leu Cys
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ctg aac cgt tat tac gga tgg tat gtc caa agc ggc gat ttg gaa acg      1440
Leu Asn Arg Tyr Tyr Gly Trp Tyr Val Gln Ser Gly Asp Leu Glu Thr
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gca gag aag gta ctg gaa aaa gaa ctt ctg gcc tgg cag gag aaa ctg      1488
Ala Glu Lys Val Leu Glu Lys Glu Leu Leu Ala Trp Gln Glu Lys Leu
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cat cag ccg att atc atc acc gaa tac ggc gtg gat acg tta gcc ggg      1536
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ctg cac tca atg tac acc gac atg tgg agt gaa gag tat cag tgt gca      1584
Leu His Ser Met Tyr Thr Asp Met Trp Ser Glu Glu Tyr Gln Cys Ala
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tgg ctg gat atg tat cac cgc gtc ttt gat cgc gtc agc gcc gtc gtc      1632
Trp Leu Asp Met Tyr His Arg Val Phe Asp Arg Val Ser Ala Val Val
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ggt gaa cag gta tgg aat ttc gcc gat ttt gcg acc tcg caa ggc ata      1680
Gly Glu Gln Val Trp Asn Phe Ala Asp Phe Ala Thr Ser Gln Gly Ile
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Leu Arg Val Gly Gly Asn Lys Lys Gly Ile Phe Thr Arg Asp Arg Lys
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ccg aag tcg gcg gct ttt ctg ctg caa aaa cgc tgg act ggc atg aac      1776
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Gly Ser Phe Asn Asp Gln Phe Ala Asp Ala Asp Ile Arg Asn Tyr Ala
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Gly Asn Val Trp Tyr Gln Arg Glu Val Phe Ile Pro Lys Gly Trp Ala
65                  70                  75                  80
Gly Gln Arg Ile Val Leu Arg Phe Asp Ala Val Thr His Tyr Gly Lys
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Pro Phe Glu Ala Asp Val Thr Pro Tyr Val Ile Ala Gly Lys Ser Val
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5 December 2002 (05.12.2002)

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Filed on 30 May 2001 (30.05.2001)
US 60/366,891 (CIP)
Filed on 21 March 2002 (21.03.2002)

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
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[Continued on next page]

(54) Title: CHROMOSOME-BASED PLATFORMS

(57) Abstract: Artificial chromosomes, including *Aces*, that have been engineered to contain available sites for site-specific, recombination-directed integration of DNA of interest are provided. These artificial chromosomes permit tractable, efficient, rational engineering of the chromosome for a variety of applications.

WO 02/097059 A3

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/17452

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/04; C12N 15/85,87,90; A01N 43/34; C12N 15/09
US CL : 536/23.1; 435/320.1,325,419,455,467; 514/44; 800/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 536/23.1; 435/320.1,325,419,455,467; 514/44; 800/21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS (EAST); STN (MEDLINE, BIOSIS, CAPLUS)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 01/07572 A2 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 01 February 2001 (01.02.2001), see entire document.	1,2,7,8,11,23-25,27,50 ----------- 3-6,9,10,12,13,26,28-
X --- Y	WO 00/11155 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 02March 2000 (02.03.2000), see entire document.	1,2,7,8,11,23-25,27,50 ----------- 3-6,9,10,12,13,26,28-
X --- Y	US 6,171,861 B1 (HARTLEY et al.) 09 January 2001 (09.01.2001), see entire document, especially sequence listing.	1-3,8,10-12,23-27,33,36,39,40,43-45,50,54,55,85 ----------- 4-7,9,13-22,28-30,32,34,35,37,38,41,42,46-49,51-53,56,67-71,84,86

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 November 2002 (01.11.2002)	27 NOV 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Daniel M Sullivan Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

PCT/US02/17452

INTERNATIONAL SEARCH REPORT

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 96/40724 A1 (LIFE TECHNOLOGIES, INC.) 19 December 1996 (19.12.96), see entire document, especially SEQ ID NO:1-16.	1-3,8,10-12,23-27,33,36,39,40,43-45,50,54,55,85 ----------- 1-3,8,10-12,23-27,33,36,39,40,43-45,50,54,55,85
Y	WO 94/00569 A1 (GENPHARM INTERNATIONAL, INC.) 06 January 1994 (06.01.94), see entire document.	72-78
Y	WO 94/23049 A2 (THE JOHNS HOPKINS UNIVERSITY) 13 October 1994 (13.10.94), see entire document.	72-78
X --- Y	US 5,721,118 A (SCHEFFLER) 24 February 1998 (24.02.98), see entire document, especially Example 3.	14,15,22-25,57 ----------- 16-21,26,58-64,72-78,84,85,98-105,123
X --- Y	US 5,948,653 A (PATI et al.) 07 September 1999 (07.09.99), see entire document, especially the first full paragraph in column 29.	14,15,22-25,57 ----------- 16-21,26,58-64,72-78,84,85,98-105,123
X --- Y	GB 2 331 752 A (MEDICAL RESEARCH COUNCIL) 02 June 1999 (02.06.99), see entire document, especially pages 8-10.	109,123 ----------- 72-78,110-122
A	HALDIMANN et al. Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure-function studies of bacteria. J Bacteriol. November 2001, Vol. 183 No. 21, pages 6384-6393, see entire document.	65,66
X,P --- Y,P	MORALLI et al. Insertion of a loxP site in a size-reduced human accessory chromosome. Cytogenet. Cell Genet. 2001, Vol. 94 No. 3-4, pages 113-120, see entire document.	91-93,106,108 ----------- 94-97,107
X --- Y	LORBACH et al. Site-specific recombination in human cells catalyzed by phage lambda integrase mutants. J. Mol. Biol. March 2000, Vol. 296 No. 5, pages 1175-1181, see entire document.	1,2,8,50 ----------- 3-7,9-13,23-32,51-56,84,85
X --- Y	CALL et al. A cre-lox recombination system for the targeted integration of circular yeast artificial chromosomes into embryonic stem cells.Hum. Mol. Genet. July 2000, Vol. 9, No. 12, pages 1745-1751, see entire document.	14,15,22-26,57,59 ----------- 16-21,58,60,84,85,98-105
Y,P	HADLACZKY, G. Satellite DNA-based artificial chromosomes for use in gene therapy. Curr. Opin. Mol. Ther. April 2001, Vol. 3 No. 2, pages 125-132, see entire document.	1-123

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/17452

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-64, 67-71, 79, 84-86, 91-108, and 123, drawn to eukaryotic recombinogenic chromosomes used for introducing heterologous nucleic acids into a chromosome and the resulting cells.
Group II, claim(s) 65-66 and 87-88, drawn to a lambda intR mutein.
Group III, claim(s) 72-78, drawn to the production of transgenic animals.
Group IV, claim(s) 80, drawn to the production of an artificial chromosome library.
Group V, claim(s) 81-83, drawn to a library of cells for genomic screening.
Group VI, claim(s) 89-90, drawn to a modified iron-induced promoter.
Group VII, claim(s) 109-122, drawn to a method for screening compounds and their effects on regulatory regions.

and it considers that the International Application does not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated below:

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of Group I which defines an advance over the art is a eukaryotic chromosome containing recombinogenic sites that can be used to introduce heterologous nucleic acids into chromosomes, and cells containing these recombinogenic chromosomes.
The special technical feature of Group II involves a lambda intR mutein. This feature defines an advance over Group I in that it involves a protein that is not required for the technical feaures as set forth above in Group I.
The special technical feature of Group III involves the production of a transgenic animal, which represents a second method for using the invention as set forth above in Group I.
The special technical feature of Group IV involves the production of artificial chromosome expression system libraries, which represents a third method for using the invention as set forth above in Group I.
The special technical feature of Group V involves a library of cells containing the artificial chromosome expression system libraries set foth above in Group IV, and represents a first product resulting from Group IV.
The special technical feature of Group VI involves a modified iron-inducible promoter. This feature defines an advance over Group I in that it involves a promoter that is not required for the technical features as set forth above in Group I.
The special technical feature of Group VII involves a method for screening compounds for their effect on regulatory regions, which represents a fourth method of using the invention as set forth above in Group I.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicant *(for all designated States except US)*: **CHROMOS MOLECULAR SYSTEMS, INC.** [CA/CA]; 8081 Lougheed Highway, Burnaby, B.C. V5A 1W9 (CA).

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(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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[Continued on next page]

(54) Title: CHROMOSOME-BASED PLATFORMS

(57) Abstract: Artificial chromosomes, including *Aces*, that have been engineered to contain available sites for site-specific, recombination-directed integration of DNA of interest are provided. These artificial chromosomes permit tractable, efficient, rational engineering of the chromosome for a variety of applications.

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Date of publication of the amended claims: 31 December 2003

(15) Information about Correction:
Previous Correction:
see PCT Gazette No. 39/2003 of 25 September 2003, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

AMENDED CLAIMS
[received by the International Bureau on 27 January 2003 (27.01.2003);
Original claims 22, 29, 39, 40, 68, 104, 107, 108, 114 and 121 replaced by
Amended Claims 22, 29, 39, 40, 68, 104, 107, 108, 114 and 121.;
Remaining claims unchanged]

13. The chromosome of claim 6 that is an artificial chromosome expression system (*ACes*).

14. A platform artificial chromosome expression system (*ACes*) comprising one or a plurality of sites that participate in recombinase catalyzed recombination.

15. The *ACes* of claim 14 that contains one site.

16. The *ACes* of claim 14 that is predominantly heterochromatin.

17. The *ACes* of claim 14 that contains no more than about 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% euchromatin.

18. The *ACes* of claim 14 that is a plant *ACes*.

19. The *ACes* of claim 14 that is an animal *ACes*.

20. The *ACes* of claim 14 that is selected from a fish, insect, reptile, amphibian, arachnid or a mammalian *ACes*.

21. The *ACes* of claim 14 that is a fish *ACes*.

22. The artificial chromosome expression system (*ACes*) of claim 14, wherein the recombinase and site(s) are from the Cre/lox system of bacteriophage P1, the int/att system of lambda phage, the FLP/FRT system of yeast, the Gin/gix recombinase system of phage Mu, the Cin recombinase system, the Pin recombinase system of *E. coli*, the R/RS system of the pSR1 plasmid, or any combination thereof.

23. A method of introducing heterologous nucleic acid into a chromosome, comprising:

contacting a chromosome of any of claims 1 or 14 with a nucleic acid molecule comprising both the heterologous nucleic acid and a recombination site, in the presence of a recombinase that promotes recombination between the sites in the chromosome and in the nucleic acid molecule.

24. The method of claim 23, wherein the recombinase is selected from the group consisting of Cre, Gin, Cin, Pin, FLP, a phage integrase and R from the pSR1 plasmid.

25. The method of claim 23, wherein the nucleic acid molecule encodes a therapeutic protein, antisense nucleic acid, or comprises an artificial chromosome.

26. The method of claim 25, wherein the nucleic acid molecule comprises a yeast artificial chromosomes (YAC), a bacterial artificial chromosome (BAC) or an insect artificial chromosome (IAC).

27. A combination, comprising, the chromosome of claim 1 and a first vector comprising the cognate recombination site, wherein the cognate recombination site is a site that recombines with the site engineered into the chromosome.

28. The combination of claim 27, further comprising nucleic acid encoding a recombinase, wherein the nucleic acid is on a second vector or on the first vector, or on the *ACes* under an inducible promoter.

29. The combination of claim 28, wherein the recombinase and sites are from the Cre/lox system of bacteriophage P1, the int/att system of lambda phage, the FLP/FRT system of yeast, the Gin/gix recombinase system of phage Mu, the Pin recombinase system of *E. coli*, the R/RS system of the pSR1 plasmid, or any combination thereof.

30. The combination of claim 28, wherein a vector is the plasmid pCXLamIntR.

31. The combination of claim 27, wherein a vector is the plasmid pDsRedN1-attB.

32. A kit, comprising the combination of claim 27 and optionally instructions for introducing heterologous nucleic acid into the chromosome.

33. A method for introducing heterologous nucleic acid into a platform artificial chromosome, comprising:

(a) mixing an artificial chromosome comprising at least a first recombination site and a vector comprising at least a second recombination site and the heterologous nucleic acid;

(b) incubating the resulting mixture in the presence of at least one recombination protein under conditions whereby recombination between the first and second recombination sites is effected, thereby introducing the heterologous nucleic acid into the artificial chromosome.

34. The method of claim 33, wherein the artificial chromosome is an *ACes*.

35. The method of claim 33, wherein said mixing step (a) is conducted in cells ex vivo.

36. The method of claim 33, wherein said mixing step (a) is conducted extracellularly in an in vitro reaction mixture.

37. The method of claim 33, wherein the at least one recombination protein is encoded by a bacteriophage selected from the group consisting of bacteriophage lambda, phi 80, P22, P2, 186, P4 and P1.

38. The method of claim 37, wherein the at least one recombination protein is encoded by bacteriophage lambda, or mutants thereof.

39. The method of claim 33, wherein at least one recombination protein is selected from the group consisting of Int, IHF, Xis, Cre, $\gamma\delta$, Tn3 resolvase, Hin, Gin, Cin and Flp.

40. The method of claim 33, wherein the recombination sites are selected from the group consisting of att and lox P sites.

66. The lambda-intR mutein of claim 65, wherein the lambda-intR mutein comprises SEQ ID NO:37.

67. The method of claim 46 wherein the promoterless marker is transcriptionally downstream of the heterologous nucleic acid, wherein the heterologous nucleic acid encodes a heterologous protein, and wherein the expression level of the selectable marker is transcriptionally linked to the expression level of the heterologous protein.

68. The method of claim 67, wherein the selectable marker and the heterologous nucleic acid are transcriptionally linked by the presence of an IRES between them.

69. The method of claim 68, wherein the selectable marker is selected from the group consisting of an antibiotic resistance gene, and a detectable protein, wherein the detectable protein is chromogenic or fluorescent.

70. The method of claim 69, wherein the selectable marker is selected from the group consisting of green fluorescent protein (GFP), red fluorescent protein (RFP), blue fluorescent protein (BFP), and *E. coli* histidinol dehydrogenase.

71. The method of claim 67 further comprising expressing the heterologous protein and isolating the heterologous protein.

72. A method for producing a transgenic animal, comprising introducing a platform-*ACes* into an embryonic cell.

73. The method of claim 72, wherein the embryonic cell is a stem cell.

74. The method of claim 72, wherein the embryonic cell is in an embryo.

75. The method of claim 72, wherein the platform-*ACes* comprises heterologous nucleic acid that encodes a therapeutic product.

a sequence of nucleotides that targets the vector to an amplifiable region of a chromosome.

92. The vector of claim 91, wherein the amplifiable region comprises heterochromatic nucleic acid.

93. The vector of claim 91, wherein the amplifiable region comprises rDNA.

94. The vector of claim 93, wherein the rDNA comprises an intergenic spacer.

95. The vector of claim 91, further comprising nucleic acid encoding a selectable marker that is not operably associated with any promoter.

96. The vector of claim 91, wherein the chromosome is a mammalian chromosome.

97. The vector of claim 91, wherein the chromosome is a plant chromosome.

98. A cell of claim 57 that is a plant cell, wherein the *ACes* platform is a MAC.

99. The plant cell of claim 98, wherein the MAC comprises transcriptional regulatory sequence of nucleotides derived from plants.

100. The plant cell of claim 99, wherein the regulatory sequence is selected from the group consisting of promoters, terminators, enhancers, silencers and transcription factor binding sites.

101. A cell of claim 57 that is an animal cell, wherein the *ACes* platform is a plant artificial chromosome (PAC).

102. The cell of claim 101 that is a mammalian cell.

103. The cell of claim 98, wherein the MAC comprises transcriptional regulatory sequence of nucleotides derived from plants.

104. The cell of claim 102, wherein the PAC comprises transcriptional regulatory sequence of nucleotides derived from animals.

105. The cell of claim 104, wherein the regulatory sequence is selected from the group consisting of promoters, terminators, enhancers, silencers and transcription factor binding sites.

106. A method, comprising:

introducing a vector of claim 91 into a cell;

growing the cells; and

selecting a cell comprising an artificial chromosome that comprises one or more repeat regions.

107. The method of claim 106, wherein a sufficient portion of the vector integrates into a chromosome in the cell to result in amplification of chromosomal DNA.

108. The method of claim 106, wherein the artificial chromosome is an *ACes*.

109. A method for screening, comprising:

contacting a cell comprising a reporter *ACes* with test compounds or known compounds, wherein:

the reporter *ACes* comprises one or a plurality of reporter constructs;

a reporter construct comprises a reporter gene in operative linkage with a regulatory region responsive to test or known compounds; and

detecting any increase or decrease in signal output from the reporter, wherein a change in the signal is indicative of activity of the test or known compound on the regulatory region.

110. The method of claim 109, wherein the reporter is operatively linked to a promoter that controls expression of a gene in a signal transduction pathway, whereby activation or reduction in the signal indicates that the pathway is activated or down-regulated by the test compound.

111. The method of claim 109, wherein the reporter in the construct encodes drug resistance or encodes a fluorescent protein.

112. The method of claim 111, wherein the fluorescent protein is selected from the group consisting of red, green and blue fluorescent proteins.

113. The method of claim 109, wherein the *ACes* comprises a plurality of reporter-linked constructs, each with a different reporter, whereby the pathway(s) affected by the test compounds can be elucidated.

114. The method of claim 109, wherein a reporter is operatively linked to a promoter that is transcriptionally regulated in response to DNA damage, and the test compounds are genotoxicants.

115. The method of claim 114, wherein the DNA damage is induced by apoptosis, necrosis or cell-cycle perturbations.

116. The method of claim 114, wherein unknown compounds are screened to assess whether they are genotoxicants.

117. The method of claim 114, wherein the promoter is a cytochrome P450-profiled promoter.

118. The method of claim 114, wherein the cell is in a transgenic animal and toxicity is assessed in the animal.

119. The method of claim 109, wherein:

the cell is a patient cell sample; the patient has a disease;

the regulatory region is one targeted by a drug or drug regimen; and

the method assesses the effectiveness of a treatment for the disease for the particular patient.

120. The method of claim 119, wherein the cell is a tumor cell.

121. The method of claim 109, wherein the cell is a stem cell or a progenitor cell, whereby expression of the reporter is operatively linked to

a regulatory region expressed in the cells to thereby identify stem cells or progenitor cells.

122. The method of claim 109, wherein the cell is in an animal; and the method comprises whole-body imaging to monitor expression of the reporter in the animal.

123. A reporter *ACes* comprises one or a plurality of reporter constructs, wherein the reporter construct comprises a reporter gene in operative linkage with a regulatory region responsive to test or known compounds.

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(54) Title: CHROMOSOME-BASED PLATFORMS

(57) Abstract: Artificial chromosomes, including *Aces*, that have been engineered to contain available sites for site-specific, recombination-directed integration of DNA of interest are provided. These artificial chromosomes permit tractable, efficient, rational engineering of the chromosome for a variety of applications.

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CHROMOSOME-BASED PLATFORMS

RELATED APPLICATIONS

Benefit of priority to U.S. provisional application Serial No. 60/294,758, filed May 30, 2001, to Perkins, *et al.*, entitled "CHROMOSOME-BASED PLATFORMS" and to U.S. provisional application Serial No. 60/366,891, filed March 21, 2002, to Perkins, *et al.*, entitled "CHROMOSOME-BASED PLATFORMS" is claimed. Where permitted, the subject matter of which are herein incorporated by reference in their entirety.

This application is related to Provisional Application No. 60/294,687, filed May 30, 2001, by CARL PEREZ AND STEVEN FABIJANSKI entitled *PLANT ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING PLANT ARTIFICIAL CHROMOSOMES* and to U.S. Provisional Application No. 60/296,329, filed June 4, 2001, by CARL PEREZ AND STEVEN FABIJANSKI entitled *PLANT ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING PLANT ARTIFICIAL CHROMOSOMES*. This application also is related to U.S. Provisional Application No. 60/294,758, filed May 30, 2001, by EDWARD PERKINS *et al.*. entitled *CHROMOSOME-BASED PLATFORMS* and to U.S. Provisional Application No. 60/366,891, filed March 21, 2002, by by EDWARD PERKINS *et al.*. entitled *CHROMOSOME-BASED PLATFORMS*. This application is also related to U.S. application Serial Nos. (attorney dkt nos. 24601-419 and 419PC), filed on the same day herewith, entitled *PLANT ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS OF PREPARING PLANT ARTIFICIAL CHROMOSOMES* to Perez *et al.*.

This application is related to U.S. application Serial No. 08/695,191, filed August 7, 1996 by GYULA HADLACZKY and ALADAR SZALAY, entitled *ARTIFICIAL CHROMOSOMES, USES THEREOF AND*

METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES, now U.S. Patent No. 6,025,155. This application is also related to U.S. application Serial No. 08/682,080, filed July 15, 1996 by GYULA HADLACZKY and ALADAR SZALAY, entitled *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES*, now U.S. Patent No. 6,077,697. This application is also related U.S. application Serial No. 08/629,822, filed April 10, 1996 by GYULA HADLACZKY and ALADAR SZALAY, entitled *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES* (now abandoned), and is also related to copending U.S. application Serial No. 09/096,648, filed June 12, 1998, by GYULA HADLACZKY and ALADAR SZALAY, entitled *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES* and to U.S. application Serial No. 09/835,682, April 10, 1997 by GYULA HADLACZKY and ALADAR SZALAY, entitled *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES* (now abandoned). This application is also related to copending U.S. application Serial No. 09/724,726, filed November 28, 2000, U.S. application Serial No. 09/724,872, filed November 28, 2000, U.S. application Serial No. 09/724,693, filed November 28, 2000, U.S. application Serial No. 09/799,462, filed March 5, 2001, U.S. application Serial No. 09/836,911, filed April 17, 2001, and U.S. application Serial No. 10/125,767, filed April 17, 2002, each of which is by GYULA HADLACZKY and ALADAR SZALAY, and is entitled *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES*. This application is also related to International PCT application No. WO 97/40183. Where permitted the

subject matter of each of these provisional applications, international applications, and applications is incorporated by reference in its entirety.

FIELD OF INVENTION

Artificial chromosomes, including *ACes*, that have been engineered to contain available sites for site-specific, recombination-directed integration of DNA of interest are provided. These artificial chromosomes permit tractable, efficient, rational engineering of the chromosome.

BACKGROUND

Artificial chromosomes

A variety of artificial chromosomes for use in plants and animals, particularly higher plants and animals are available. In particular, U.S. Patent Nos. 6,025,155 and 6,077,697 provide heterochromatic artificial chromosomes designated therein as satellite artificial chromosomes (SATACs) and now designated artificial chromosome expression systems (*ACes*). These chromosomes are prepared by introducing heterologous DNA into a selected plant or animal cell under conditions that result in integration into a region of the chromosome that leads to an amplification event resulting in production of a dicentric chromosome. Subsequent treatment and growth of cells with dicentric chromosomes, including further amplifications, ultimately results in the artificial chromosomes provided therein. In order to introduce a desired heterologous gene (or a plurality of heterologous genes) into the artificial chromosome, the process is repeated introducing the desired heterologous genes and nucleic acids in the initial targeting step. This process is time consuming and tedious. Hence, more tractable and efficient methods for introducing heterologous nucleic acid molecules into artificial chromosomes, particularly *ACes*, are needed.

Therefore, it is an object herein to provide engineered artificial chromosomes that permit tractable, efficient and rational engineering of artificial chromosomes.

SUMMARY OF THE INVENTION

Provided herein are artificial chromosomes that permit tractable, efficient and rational engineering thereof. In particular, the artificial chromosomes provided herein contain one or a plurality of loci (sites) for site-specific, recombination-directed integration of DNA. Thus, provided herein are platform artificial chromosome expression systems ("platform *ACes*") containing single or multiple site-specific, recombination sites. The artificial chromosomes and *ACes* artificial chromosomes include plant and animal chromosomes. Any recombinase system that effects site-specific recombination is contemplated for use herein.

In one embodiment, chromosomes, including platform *ACes*, are provided that contain one or more lambda *att* sites designed for recombination-directed integration in the presence of lambda integrase, and that are mutated so that they do not require additional factors. Methods for preparing such chromosomes, vectors for use in the methods, and uses of the resulting chromosomes are also provided.

Platform *ACes* containing the recombination site(s) and methods for introducing heterologous nucleic acid into such sites and vectors therefor, are provided.

Also provided herein is a bacteriophage lambda (λ) integrase site-specific recombination system.

Methods using recombinase mediated recombination target gene expression vectors and/or genes for insertion thereof into platform chromosomes and the resulting chromosomes are provided.

Combinations and kits containing the combinations of vectors encoding a recombinase and integrase and primers for introduction of the

site recognized thereby are also provided. The kits optionally include instructions for performing site-directed integration or preparation of *ACes* containing such sites.

Also provided herein are mammalian and plant cells comprising the artificial chromosomes and *ACes* described herein. The cells can be nuclear donor cells, stem cells, such as a mesenchymal stem cell, a hematopoietic stem cell, an adult stem cell or an embryonic stem cell.

Also provided is a lamba-intR mutein comprising a glutamic acid to arginine change at position 174 of wild-type lambda-integrase3. Also provided are transgenic animals and methods for producing a transgenic animal, comprising introducing a *ACes* into an embryonic cell, such as a stem cell or embryo. The *ACes* can comprise heterologous nucleic acid that encodes a therapeutic product. The transgenic animal can be a fish, insect, reptile, amphibians, arachnid or mammal. In certain embodiments, the *ACes* is introduced by cell fusion, lipid-mediated transfection by a carrier system, microinjection, microcell fusion, electroporation, microprojectile bombardment or direct DNA transfer.

The platform *ACes*, including plant and animal *ACes*, such as MACs, provided herein can be introduced into cells, such as, but not limited to, animal cells, including mammalian cells, and into plant cells. Hence plant cells that contain platform MACs, animal cells that contain platform PACs and other combinations of cells and platform *ACes* are provided.

DESCRIPTION OF FIGURES

FIGURE 1 provides a diagram depicting creation of an exemplary *ACes* artificial chromosome prepared using methods detailed in U.S. Patent Nos. 6,025,155 and 6,077,697 and International PCT application No. WO 97/40183. In this exemplified embodiment, the nucleic acid is targeted to an acrocentric chromosome in an animal or plant, and the

heterologous nucleic acid includes a sequence-specific recombination site and marker genes.

FIGURE 2 provides a map of pWEPuro9K, which is a targeting vector derived from the vector pWE15 (GenBank Accession # X65279; SEQ ID No. 31). Plasmid pWE15 was modified by replacing the *Sal*I (Klenow filled)/*Sma*I neomycin resistance encoding fragment with the *Pvu*II/*Bam*HI (Klenow filled) puromycin resistance-encoding fragment (isolated from plasmid pPUR, Clontech Laboratories, Inc., Palo Alto, CA; GenBank Accession no. U07648; SEQ ID No. 30) resulting in plasmid pWEPuro. Subsequently a 9 Kb *Not*I fragment from the plasmid pFK161 (see Example 1, see, also Csonka *et al.* (2000) *Journal of Cell Science 113*:3207-32161; and SEQ ID NO: 118), containing a portion of the mouse rDNA region, was cloned into the *Not*I site of pWEPuro resulting in plasmid pWEPuro9K.

FIGURE 3 depicts construction of an *ACes* platform chromosome with a single recombination site, such as loxP sites or an *att*P or *att*B site. This platform *ACes* chromosome is an exemplary artificial chromosome with a single recombination site.

FIGURE 4 provides a map of plasmid pSV40-193attPsensePur.

FIGURE 5 depicts a method for formation of a chromosome platform with multiple recombination integration sites, such as attP sites.

FIGURE 6 sets forth the sequences of the core region of *att*P, *att*B, *att*L and *att*R (SEQ ID Nos. 33-36).

FIGURE 7 depicts insertional recombination of a vector encoding a marker gene, DsRed and an *att*B site with an artificial chromosome containing an *att*P site.

FIGURE 8 provides a map of plasmid pCXLamIntR (SEQ ID NO: 112), which includes the Lambda integrase (E174R)-encoding nucleic acid.

FIGURE 9 diagrammatically summarizes the platform technology; marker 1 permits selection of the artificial chromosomes containing the integration site; marker 2, which is promoterless in the target gene expression vector, permits selection of recombinants. Upon recombination with the platform marker 2 is expressed under the control of a promoter resident on the platform.

FIGURE 10 provides the vector map for the plasmid p18attBZEO-5'6XHS4eGFP (SEQ ID NO: 116).

FIGURE 11 provides the vector map for the plasmid p18attBZEO-3'6XHS4eGFP (SEQ ID NO: 115).

FIGURE 12 provides the vector map for the plasmid p18attBZEO-(6XHS4)2eGFP (SEQ ID NO: 110).

FIGURES 13 AND 14 depict the integration of a PCR product by site-specific recombination as set forth in Example 8.

FIGURE 15 provides the vector map for the plasmid pPACrDNA as set forth in Example 9.A.

DETAILED DESCRIPTION OF THE INVENTION

A. DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention(s) belong. All patents, patent applications, published applications and publications, Genbank sequences, websites and other published materials referred to throughout the entire disclosure herein, unless noted otherwise, are incorporated by reference in their entirety. Where reference is made to a URL or other such indentifier or address, it understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference thereto evidences the availability and public dissemination of such information.

As used herein, nucleic acid refers to single-stranded and/or double-stranded polynucleotides, such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), as well as analogs or derivatives of either RNA or DNA. Also included in the term "nucleic acid" are analogs of nucleic acids such as peptide nucleic acid (PNA), phosphorothioate DNA, and other such analogs and derivatives. When referring to probes or primers, optionally labeled, with a detectable label, such as a fluorescent or radiolabel, single-stranded molecules are contemplated. Such molecules are typically of a length such that they are statistically unique and of low copy number (typically less than 5, preferably less than 3) for probing or priming a library. Generally a probe or primer contains at least 14, 16 or 30 contiguous nucleotides of sequence complementary to or identical to a gene of interest. Probes and primers can be 10, 20, 30, 50, 100 or more nucleotides long.

As used herein, DNA is meant to include all types and sizes of DNA molecules including cDNA, plasmids and DNA including modified nucleotides and nucleotide analogs.

As used herein, nucleotides include nucleoside mono-, di-, and triphosphates. Nucleotides also include modified-nucleotides, such as, but are not limited to, phosphorothioate nucleotides and deazapurine nucleotides and other nucleotide analogs.

As used herein, heterologous or foreign DNA and RNA are used interchangeably and refer to DNA or RNA that does not occur naturally as part of the genome in which it is present or which is found in a location or locations and/or in amounts in a genome or cell that differ from that in which it occurs in nature. Heterologous nucleic acid is generally not endogenous to the cell into which it is introduced, but has been obtained from another cell or prepared synthetically. Generally, although not necessarily, such nucleic acid encodes RNA and proteins that are not

normally produced by the cell in which it is expressed. Any DNA or RNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which it is expressed is herein encompassed by heterologous DNA. Heterologous DNA and RNA may also encode RNA or proteins that mediate or alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes.

Examples of heterologous DNA include, but are not limited to, DNA that encodes a gene product or gene product(s) of interest, introduced for purposes of modification of the endogenous genes or for production of an encoded protein. For example, a heterologous or foreign gene may be isolated from a different species than that of the host genome, or alternatively, may be isolated from the host genome but operably linked to one or more regulatory regions which differ from those found in the unaltered, native gene. Other examples of heterologous DNA include, but are not limited to, DNA that encodes traceable marker proteins, such as a protein that confers traits including, but not limited to, herbicide, insect, or disease resistance; traits, including, but not limited to, oil quality or carbohydrate composition. Antibodies that are encoded by heterologous DNA may be secreted or expressed on the surface of the cell in which the heterologous DNA has been introduced.

As used herein, operative linkage or operative association, or grammatical variations thereof, of heterologous DNA to regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences refers to the relationship between such DNA and such sequences of nucleotides. For example, operative linkage of heterologous DNA to a promoter refers to the physical relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the

promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA.

In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation (*i.e.*, start) codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, *e.g.*, Kozak (1991) *J. Biol. Chem.* *266*:19867-19870) can be inserted immediately 5' of the start codon and may enhance expression.

As used herein, a sequence complementary to at least a portion of an RNA, with reference to antisense oligonucleotides, means a sequence having sufficient complementarity to be able to hybridize with the RNA, preferably under moderate or high stringency conditions, forming a stable duplex. The ability to hybridize depends on the degree of complementarity and the length of the antisense nucleic acid. The longer the hybridizing nucleic acid, the more base mismatches it can contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

As used herein, regulatory molecule refers to a polymer of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) or a polypeptide that is capable of enhancing or inhibiting expression of a gene.

As used herein, recognition sequences are particular sequences of nucleotides that a protein, DNA, or RNA molecule, or combinations thereof, (such as, but not limited to, a restriction endonuclease, a modification methylase and a recombinase) recognizes and binds. For example, a recognition sequence for Cre recombinase (see, *e.g.*, SEQ ID NO:58) is a 34 base pair sequence containing two 13 base pair inverted

repeats (serving as the recombinase binding sites) flanking an 8 base pair core and designated loxP (see, *e.g.*, Sauer (1994) *Current Opinion in Biotechnology* *5*:521-527). Other examples of recognition sequences, include, but are not limited to, attB and attP, *att*R and *att*L and others (see, *e.g.*, SEQ ID Nos. 8, 41-56 and 72), that are recognized by the recombinase enzyme Integrase (see, SEQ ID Nos. 37 and 38 for the nucleotide and encoded amino acid sequences of an exemplary lambda phage integrase).

The recombination site designated *att*B is an approximately 33 base pair sequence containing two 9 base pair core-type Int binding sites and a 7 base pair overlap region; *att*P (SEQ ID No. 72) is an approximately 240 base pair sequence containing core-type Int binding sites and arm-type Int binding sites as well as sites for auxiliary proteins IHF, FIS, and Xis (see, *e.g.*, Landy (1993) *Current Opinion in Biotechnology* *3*:699-707l see, *e.g.*, SEQ ID Nos. 8 and 72).

As used herein, a recombinase is an enzyme that catalyzes the exchange of DNA segments at specific recombination sites. An integrase herein refers to a recombinase that is a member of the lambda (λ) integrase family.

As used herein, recombination proteins include excisive proteins, integrative proteins, enzymes, co-factors and associated proteins that are involved in recombination reactions using one or more recombination sites (see, Landy (1993) *Current Opinion in Biotechnology* *3*:699-707). The recombination proteins used herein can be delivered to a cell via an expression cassette on an appropriate vector, such as a plasmid, and the like. In other embodiments, the recombination proteins can be delivered to a cell in protein form in the same reaction mixture used to deliver the desired nucleic acid, such as a platform *ACes*, donor target vectors, and the like.

As used herein the expression "lox site" means a sequence of nucleotides at which the gene product of the cre gene, referred to herein as Cre, can catalyze a site-specific recombination event. A LoxP site is a 34 base pair nucleotide sequence from bacteriophage P1 (see, *e.g.*, Hoess *et al.* (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*:3398-3402). The LoxP site contains two 13 base pair inverted repeats separated by an 8 base pair spacer region as follows: (SEQ ID NO. 57):

ATAACTTCGTATA ATGTATGC TATACGAAGTTAT

*E. coli*DH5Δlac and yeast strain BSY23 transformed with plasmid pBS44 carrying two loxP sites connected with a LEU2 gene are available from the American Type Culture Collection (ATCC) under accession numbers ATCC 53254 and ATCC 20773, respectively. The lox sites can be isolated from plasmid pBS44 with restriction enzymes *Eco*RI and *Sal*I, or *Xho*I and *Bam*HI. In addition, a preselected DNA segment can be inserted into pBS44 at either the *Sal*I or *Bam*HI restriction enzyme sites. Other lox sites include, but are not limited to, LoxB, LoxL, LoxC2 and LoxR sites, which are nucleotide sequences isolated from *E. coli* (see, *e.g.*, Hoess *et al.* (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*:3398). Lox sites can also be produced by a variety of synthetic techniques (see, *e.g.*, Ito *et al.* (1982) *Nuc. Acid Res. 10:*1755 and Ogilvie *et al.* (1981) *Science 270*:270).

As used herein, the expression "cre gene" means a sequence of nucleotides that encodes a gene product that effects site-specific recombination of DNA in eukaryotic cells at lox sites. One cre gene can be isolated from bacteriophage P1 (see, *e.g.*, Abremski *et al.* (1983) *Cell 32*:1301-1311). *E. coli* DH1 and yeast strain BSY90 transformed with plasmid pBS39 carrying a cre gene isolated from bacteriophage P1 and a GAL1 regulatory nucleotide sequence are available from the American Type Culture Collection (ATCC) under accession numbers ATCC 53255

and ATCC 20772, respectively. The cre gene can be isolated from plasmid pBS39 with restriction enzymes *XhoI* and *SalI*.

As used herein, site-specific recombination refers to site-specific recombination that is effected between two specific sites on a single nucleic acid molecule or between two different molecules that requires the presence of an exogenous protein, such as an integrase or recombinase.

For example, Cre-lox site-specific recombination can include the following three events:

a. deletion of a pre-selected DNA segment flanked by lox sites;

b. inversion of the nucleotide sequence of a pre-selected DNA segment flanked by lox sites; and

c. reciprocal exchange of DNA segments proximate to lox sites located on different DNA molecules.

This reciprocal exchange of DNA segments can result in an integration event if one or both of the DNA molecules are circular. DNA segment refers to a linear fragment of single- or double-stranded deoxyribonucleic acid (DNA), which can be derived from any source. Since the lox site is an asymmetrical nucleotide sequence, two lox sites on the same DNA molecule can have the same or opposite orientations with respect to each other. Recombination between lox sites in the same orientation results in a deletion of the DNA segment located between the two lox sites and a connection between the resulting ends of the original DNA molecule. The deleted DNA segment forms a circular molecule of DNA. The original DNA molecule and the resulting circular molecule each contain a single lox site. Recombination between lox sites in opposite orientations on the same DNA molecule result in an inversion of the nucleotide sequence of the DNA segment located between the two lox

sites. In addition, reciprocal exchange of DNA segments proximate to lox sites located on two different DNA molecules can occur. All of these recombination events are catalyzed by the gene product of the cre gene. Thus, the Cre-lox system can be used to specifically delete, invert, or insert DNA. The precise event is controlled by the orientation of lox DNA sequences, in *cis* the lox sequences direct the *Cre* recombinase to either delete (lox sequences in direct orientation) or invert (lox sequences in inverted orientation) DNA flanked by the sequences, while *in trans* the lox sequences can direct a homologous recombination event resulting in the insertion of a recombinant DNA.

As used herein, a chromosome is a nucleic acid molecule, and associated proteins, that is capable of replication and segregation within a cell upon cell division. Typically, a chromosome contains a centromeric region, replication origins, telomeric regions and a region of nucleic acid between the centromeric and telomeric regions.

As used herein, a centromere is any nucleic acid sequence that confers an ability to segregate to daughter cells through cell division. A centromere may confer stable segregation of a nucleic acid sequence, including an artificial chromosome containing the centromere, through mitotic or meiotic divisions, including through both mitotic and meiotic divisions. A particular centromere is not necessarily derived from the same species in which it is introduced, but has the ability to promote DNA segregation in cells of that species.

As used herein, euchromatin and heterochromatin have their recognized meanings. Euchromatin refers to chromatin that stains diffusely and that typically contains genes, and heterochromatin refers to chromatin that remains unusually condensed and that has been thought to be transcriptionally inactive. Highly repetitive DNA sequences (satellite DNA) are usually located in regions of the heterochromatin surrounding

the centromere (pericentric or pericentromeric heterochromatin). Constitutive heterochromatin refers to heterochromatin that contains the highly repetitive DNA which is constitutively condensed and genetically inactive.

As used herein, an acrocentric chromosome refers to a chromosome with arms of unequal length.

As used herein, endogenous chromosomes refer to genomic chromosomes as found in a cell prior to generation or introduction of an artificial chromosome.

As used herein, artificial chromosomes are nucleic acid molecules, typically DNA, that stably replicate and segregate alongside endogenous chromosomes in cells and have the capacity to accommodate and express heterologous genes contained therein. It has the capacity to act as a gene delivery vehicle by accommodating and expressing foreign genes contained therein. A mammalian artificial chromosome (MAC) refers to chromosomes that have an active mammalian centromere(s). Plant artificial chromosomes, insect artificial chromosomes and avian artificial chromosomes refer to chromosomes that include centromeres that function in plant, insect and avian cells, respectively. A human artificial chromosome (HAC) refers to chromosomes that include centromeres that function in human cells. For exemplary artificial chromosomes, see, *e.g.*, U.S. Patent Nos. 6,025,155; 6,077,697; 5,288,625; 5,712,134; 5,695,967; 5,869,294; 5,891,691 and 5,721,118 and published International PCT application Nos, WO 97/40183 and WO 98/08964. Artificial chromosomes include those that are predominantly heterochromatic (formerly referred to as satellite artificial chromosomes (SATACs); see, *e.g.*, U.S. Patent Nos. 6,077,697 and 6,025,155 and published International PCT application No. WO 97/40183), minichromosomes that contain a *de novo* centromere (see, U.S. Patent

Nos. 5,712,134, 5,891,691 and 5,288,625), artificial chromosomes predominantly made up of repeating nucleic acid units and that contain substantially equivalent amounts of euchromatic and heterochromatic DNA and *in vitro* assembled artificial chromosomes (see, copending U.S. provisional application Serial No. 60/294,687, filed on May 30, 2001).

As used herein, the term "satellite DNA-based artificial chromosome (SATAC)" is interchangable with the term "artificial chromosome expression system (*ACes*)". These artificial chromosomes (*ACes*) include those that are substantially all neutral non-coding sequences (heterochromatin) except for foreign heterologous, typically gene-encoding nucleic acid, that is interspersed within the heterochromatin for the expression therein (see U.S. Patent Nos. 6,025,155 and 6,077,697 and International PCT application No. WO 97/40183), or that is in a single locus as provided herein. Also included are *ACes* that may include euchromatin and that result from the process described in U.S. Patent Nos. 6,025,155 and 6,077,697 and International PCT application No. WO 97/40183 and outlined herein. The delineating structural feature is the presence of repeating units, that are generally predominantly heterochromatin. The precise structure of the *ACes* will depend upon the structure of the chromosome in which the initial amplification event occurs; all share the common feature of including a defined pattern of repeating units. Generally *ACes* have more heterochromatin than euchromatin. Foreign nucleic acid molecules (heterologous genes) contained in these artificial chromosome expression systems can include any nucleic acid whose expression is of interest in a particular host cell. Such foreign nucleic acid molecules, include, but are not limited to, nucleic acid that encodes traceable marker proteins (reporter genes), such as fluorescent proteins, such as green, blue or red fluorescent proteins (GFP, BFP and RFP, respectively), other reporter

genes, such as β-galactosidase and proteins that confer drug resistance, such as a gene encoding hygromycin-resistance. Other examples of heterologous nucleic acid molecules include, but are not limited to, DNA that encodes therapeutically effective substances, such as anti-cancer agents, enzymes and hormones, DNA that encodes other types of proteins, such as antibodies, and DNA that encodes RNA molecules (such as antisense or siRNA molecules) that are not translated into proteins.

As used herein, an artificial chromosome platform, also referred to herein as a "platform *ACes*" or "*ACes* platform", refers to an artificial chromosome that has been engineered to include one or more sites for site-specific, recombination-directed integration. In particular, *ACes* that are so-engineered are provided. Any sites, including but not limited to any described herein, that are suitable for such integration are contemplated. Plant and animal platform *ACes* are provided. Among the *ACes* contemplated herein are those that are predominantly heterochromatic (formerly referred to as satellite artificial chromosomes (SATACs); see, *e.g.*, U.S. Patent Nos. 6,077,697 and 6,025,155 and published International PCT application No. WO 97/40183), artificial chromosomes predominantly made up of repeating nucleic acid units and that contain substantially equivalent amounts of euchromatic and heterochromatic DNA resulting from an amplification event depicted in the referenced patent and herein. Included among the *ACes* for use in generating platforms, are artificial chromosomes that introduce and express heterologous nucleic acids in plants (see, copending U.S. provisional application Serial No. 60/294,687, filed on May 30, 2001). These include artificial chromosomes that have a centromere derived from a plant, and, also, artificial chromosomes that have centromeres that may be derived from other organisms but that function in plants.

As used herein a "reporter *ACes*" refers to an *ACes* that comprises one or a plurality of reporter constructs, where the reporter construct comprises a reporter gene in operative linkage with a regulatory region responsive to test or known compounds.

As used herein, amplification, with reference to DNA, is a process in which segments of DNA are duplicated to yield two or multiple copies of substantially similar or identical or nearly identical DNA segments that are typically joined as substantially tandem or successive repeats or inverted repeats.

As used herein, amplification-based artificial chromosomes are artificial chromosomes derived from natural or endogenous chromosomes by virtue of an amplification event, such as one initiated by introduction of heterologous nucleic acid into rDNA in a chromosome. As a result of such an event, chromosomes and fragments thereof exhibiting segmented or repeating patterns arise. Artificial chromosomes can be formed from these chromosomes and fragments. Hence, amplification-based artificial chromosomes refer to engineered chromosomes that exhibit an ordered segmentation that is not observed in naturally occurring chromosomes and that distinguishes them from naturally occurring chromosomes. The segmentation, which can be visualized using a variety of chromosome analysis techniques known to those of skill in the art, correlates with the structure of these artificial chromosomes. In addition to containing one or more centromeres, the amplification-based artificial chromosomes, throughout the region or regions of segmentation are predominantly made up of nucleic acid units also referred to as "amplicons", that is (are) repeated in the region and that have a similar gross structure. Repeats of an amplicon tend to be of similar size and share some common nucleic acid sequences. For example, each repeat of an amplicon may contain a replication site involved in amplification of chromosome segments and/or

some heterologous nucleic acid that was utilized in the initial production of the artificial chromosome. Typically, the repeating units are substantially similar in nucleic acid composition and may be nearly identical.

The amplification-based artificial chromosomes differ depending on the chromosomal region that has undergone amplification in the process of artificial chromosome formation. The structures of the resulting chromosomes can vary depending upon the initiating event and/or the conditions under which the heterologous nucleic acid is introduced, including modification to the endogenous chromosomes. For example, in some of the artificial chromosomes provided herein, the region or regions of segmentation may be made up predominantly of heterochromatic DNA. In other artificial chromosomes provided herein, the region or regions of segmentation may be made up predominantly of euchromatic DNA or may be made up of similar amounts of heterochromatic and euchromatic DNA.

As used herein an amplicon is a repeated nucleic acid unit. In some of the artificial chromosomes described herein, an amplicon may contain a set of inverted repeats of a megareplicon. A megareplicon represents a higher order replication unit. For example, with reference to some of the predominantly heterochromatic artificial chromosomes, the megareplicon can contain a set of tandem DNA blocks (*e.g.*, ~7.5 Mb DNA blocks) each containing satellite DNA flanked by non-satellite DNA or may be made up of substantially rDNA. Contained within the megareplicon is a primary replication site, referred to as the megareplicator, which may be involved in organizing and facilitating replication of the pericentric heterochromatin and possibly the centromeres. Within the megareplicon there may be smaller (*e.g.*, 50-300 kb) secondary replicons.

In artificial chromosomes, such as those provided U.S. Patent Nos. 6,025,155 and 6,077,697 and International PCT application No. WO 97/40183, the megareplicon is defined by two tandem blocks (~7.5 Mb DNA blocks in the chromosomes provided therein). Within each artificial chromosome or among a population thereof, each amplicon has the same gross structure but may contain sequence variations. Such variations will arise as a result of movement of mobile genetic elements, deletions or insertions or mutations that arise, particularly in culture. Such variation does not affect the use of the artificial chromosomes or their overall structure as described herein.

As used herein, amplifiable, when used in reference to a chromosome, particularly the method of generating artificial chromosomes provided herein, refers to a region of a chromosome that is prone to amplification. Amplification typically occurs during replication and other cellular events involving recombination (*e.g.*, DNA repair). Such regions include regions of the chromosome that contain tandem repeats, such as satellite DNA, rDNA, and other such sequences.

As used herein, a dicentric chromosome is a chromosome that contains two centromeres. A multicentric chromosome contains more than two centromeres.

As used herein, a formerly dicentric chromosome is a chromosome that is produced when a dicentric chromosome fragments and acquires new telomeres so that two chromosomes, each having one of the centromeres, are produced. Each of the fragments is a replicable chromosome. If one of the chromosomes undergoes amplification of primarily euchromatic DNA to produce a fully functional chromosome that is predominantly (at least more than 50%) euchromatin, it is a minichromosome. The remaining chromosome is a formerly dicentric chromosome. If one of the chromosomes undergoes amplification,

whereby heterochromatin (such as, for example, satellite DNA) is amplified and a euchromatic portion (such as, for example, an arm) remains, it is referred to as a sausage chromosome. A chromosome that is substantially all heterochromatin, except for portions of heterologous DNA, is called a predominantly heterochromatic artificial chromosome. Predominantly heterochromatic artificial chromosomes can be produced from other partially heterochromatic artificial chromosomes by culturing the cell containing such chromosomes under conditions such as BrdU treatment that destabilize the chromosome and/or growth under selective conditions so that a predominantly heterochromatic artificial chromosome is produced. For purposes herein, it is understood that the artificial chromosomes may not necessarily be produced in multiple steps, but may appear after the initial introduction of the heterologous DNA. Typically, artificial chromosomes appear after about 5 to about 60, or about 5 to about 55, or about 10 to about 55 or about 25 to about 55 or about 35 to about 55 cell doublings after initiation of artificial chromosome generation, or they may appear after several cycles of growth under selective conditions and BrdU treatment.

As used herein, an artificial chromosome that is predominantly heterochromatic (*i.e.*, containing more heterochromatin than euchromatin, typically more than about 50%, more than about 70%, or more than about 90% heterochromatin) may be produced by introducing nucleic acid molecules into cells, such as, for example, animal or plant cells, and selecting cells that contain a predominantly heterochromatic artificial chromosome. Any nucleic acid may be introduced into cells in such methods of producing the artificial chromosomes. For example, the nucleic acid may contain a selectable marker and/or optionally a sequence that targets nucleic acid to the pericentric, heterochromatic region of a chromosome, such as in the short arm of acrocentric chromosomes and

nucleolar organizing regions. Targeting sequences include, but are not limited to, lambda phage DNA and rDNA for production of predominantly heterochromatic artificial chromosomes in eukaryotic cells.

After introducing the nucleic acid into cells, a cell containing a predominantly heterochromatic artificial chromosome is selected. Such cells may be identified using a variety of procedures. For example, repeating units of heterochromatic DNA of these chromosomes may be discerned by G-banding and/or fluorescence in situ hybridization (FISH) techniques. Prior to such analyses, the cells to be analyzed may be enriched with artificial chromosome-containing cells by sorting the cells on the basis of the presence of a selectable marker, such as a reporter protein, or by growing (culturing) the cells under selective conditions. It is also possible, after introduction of nucleic acids into cells, to select cells that have a multicentric, typically dicentric, chromosome, a formerly multicentric (typically dicentric) chromosome and/or various heterochromatic structures, such as a megachromosome and a sausage chromosome, that contain a centromere and are predominantly heterochromatic and to treat them such that desired artificial chromosomes are produced. Cells containing a new chromosome are selected. Conditions for generation of a desired structure include, but are not limited to, further growth under selective conditions, introduction of additional nucleic acid molecules and/or growth under selective conditions and treatment with destabilizing agents, and other such methods (see International PCT application No. WO 97/40183 and U.S. Patent Nos. 6,025,155 and 6,077,697).

As used herein, a "selectable marker" is a nucleic acid segment, generally DNA, that allows one to select for or against a molecule or a cell that contains it, often under particular conditions. These markers can encode an activity, such as, but not limited to, production of RNA,

peptide, or protein, or can provide a binding site for RNA, peptides, proteins, inorganic and organic compounds and compositions. Examples of selectable markers include but are not limited to: (1) nucleic acid segments that encode products that provide resistance against otherwise toxic compounds (e.g., antibiotics); (2) nucleic acid segments that encode products that are otherwise lacking in the recipient cell (e.g., tRNA genes, auxotrophic markers); (3) nucleic acid segments that encode products that suppress the activity of a gene product; (4) nucleic acid segments that encode products that can be identified, such as phenotypic markers, including β-galactosidase, red, blue and/or green fluorescent proteins (FPs), and cell surface proteins; (5) nucleic acid segments that bind products that are otherwise detrimental to cell survival and/or function; (6) nucleic acid segments that otherwise inhibit the activity of any of the nucleic acid segments described in Nos. 1-5 above (e.g., antisense oligonucleotides or siRNA molecules for use in RNA interference); (7) nucleic acid segments that bind products that modify a substrate (e.g. restriction endonucleases); (8) nucleic acid segments that can be used to isolate a desired molecule (e.g. specific protein binding sites); (9) nucleic acid segments that encode a specific nucleotide sequence that can be otherwise non-functional, such as for PCR amplification of subpopulations of molecules; and/or (10) nucleic acid segments, which when absent, directly or indirectly confer sensitivity to particular compounds. Thus, for example, selectable markers include nucleic acids encoding fluorescent proteins, such as green fluorescent proteins, β-galactosidase and other readily detectable proteins, such as chromogenic proteins or proteins capable of being bound by an antibody and FACs sorted. Selectable markers such as these, which are not required for cell survival and/or proliferation in the presence of a selection agent, are also referred to herein as reporter molecules. Other selectable markers, *e.g.*, the

neomycin phosphotransferase gene, provide for isolation and identification of cells containing them by conferring properties on the cells that make them resistant to an agent, *e.g.*, a drug such as an antibiotic, that inhibits proliferation of cells that do not contain the marker.

As another example, interference of gene expression by double stranded RNA has been shown in Caenorhabditis elegans, plants, Drosophila, protozoans and mammals. This method is known as RNA interference (RNAi) and utilizes short, double-stranded RNA molecules (siRNAs). The siRNAs are generally composed of a 19-22bp double-stranded RNA stem, a loop region and a 1-4 bp overhang on the 3' end. The reduction of gene expression has been accomplished by direct introduction of the siRNAs into the cell (Harborth J et al., 2001, J Cell Sci 114(pt 24):4557-65) as well as the introduction of DNA encoding and expressing the siRNA molecule. The encoded siRNA molecules are under the regulation of an RNA polymerase III promoter (see, e.g., Yu et al., 2002, Proc Natl Acad Sci USA 99(9);6047-52; Brummelkamp et al., 2002, Science 296(5567):550-3; Miyagishi et al., 2002, Nat Biotechnol 20(5):497-500; and the like). In certain embodiments, RNAi in mammalian cells may have advantages over other therapeutic methods. For example, producing siRNA molecules that block viral genetic activities in infected cells may reduce the effects of the virus. Platform *ACes* provided herein encoding siRNA molecule(s) are an additional utilization of the platform *ACes* technology. The platform *ACes* could be engineered to encode one or more siRNA molecules to create gene "knockdowns". In one embodiment, a platform *ACes* can be engineered to encode both the siRNA molecule and a replacement gene. For example, a mouse model or cell culture system could be generated using a platform *ACes* that has a knockdown of the endogenous mouse gene, by siRNA, and the human gene homolog expressing in place of the mouse gene. The placement of

siRNA encoding sequences under the regulation of a regulatable or inducible promoter would allow one to temporally and/or spatially control the knockdown effect of the corresponding gene.

As used herein, a reporter gene includes any gene that expresses a detectable gene product, which may be RNA or protein. Generally reporter genes are readily detectable. Examples of reporter genes include, but are not limited to nucleic acid encoding a fluorescent protein, CAT (chloramphenicol acetyl transferase) (Alton *et al.* (1979) *Nature 282*: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet *et al.* (1987) *Mol. Cell. Biol.* 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*:4154-4158; Baldwin *et al.* (1984) *Biochemistry 23*:3663-3667); and alkaline phosphatase (Toh *et al.* (1989) *Eur. J. Biochem. 182*:231-238, Hall *et al.* (1983) *J. Mol. Appl. Gen. 2*:101).

As used herein, growth under selective conditions means growth of a cell under conditions that require expression of a selectable marker for survival.

As used herein, an agent that destabilizes a chromosome is any agent known by those skilled in the art to enhance amplification events, and/or mutations. Such agents, which include BrdU, are well known to those skilled in the art.

In order to generate an artificial chromosome containing a particular heterologous nucleic acid of interest, it is possible to include the nucleic acid in the nucleic acid that is being introduced into cells to initiate production of the artificial chromosome. Thus, for example, a nucleic acid can be introduced into a cell along with nucleic acid encoding a selectable marker and/or a nucleic acid that targets to a heterochromatic region of a chromosome. For introducing a heterologous nucleic acid into

the cell, it can be included in a fragment that includes a selectable marker or as part of a separate nucleic acid fragment and introduced into the cell with a selectable marker during the process of generating the artificial chromosomes. Alternatively, heterologous nucleic acid can be introduced into an artificial chromosome at a later time after the initial generation of the artificial chromosome.

As used herein, the minichromosome refers to a chromosome derived from a multicentric, typically dicentric, chromosome that contains more euchromatic than heterochromatic DNA. For purposes herein, the minichromosome contains a *de novo* centromere (e.g., a neocentromere). In some embodiments, for example, the minichromosome contains a centromere that replicates in animals, e.g., a mammalian centromere or in plants, e.g., a plant centromere.

As used herein, *in vitro* assembled artificial chromosomes or synthetic chromosomes can be either more euchromatic than heterochromatic or more heterochromatic than euchromatic and are produced by joining essential components of a chromosome *in vitro*. These components include at least a centromere, a megareplicator, a telomere and optionally secondary origins of replication.

As used herein, *in vitro* assembled plant or animal artificial chromosomes are produced by joining essential components (at least the centromere, telomere(s), megareplicator and optional secondary origins of replication) that function in plants or animals. In particular embodiments, the megareplicator contains sequences of rDNA, particularly plant or animal rDNA.

As used herein, a plant is a eukaryotic organism that contains, in addition to a nucleus and mitochondria, chloroplasts capable of carrying out photosynthesis. A plant can be unicellular or multicellular and can contain multiple tissues and/or organs. Plants can reproduce sexually or

asexually and can be perennial or annual in growth. Plants can also be terrestrial or aquatic. The term "plant" includes a whole plant, plant cell, plant protoplast, plant calli, plant seed, plant organ, plant tissue, and other parts of a whole plant.

As used herein, stable maintenance of chromosomes occurs when at least about 85%, preferably 90%, more preferably 95%, of the cells retain the chromosome. Stability is measured in the presence of a selective agent. Preferably these chromosomes are also maintained in the absence of a selective agent. Stable chromosomes also retain their structure during cell culturing, suffering no unintended intrachromosomal or interchromosomal rearrangements.

As used herein, *de novo* with reference to a centromere, refers to generation of an excess centromere in a chromosome as a result of incorporation of a heterologous nucleic acid fragment using the methods herein.

As used herein, BrdU refers to 5-bromodeoxyuridine, which during replication is inserted in place of thymidine. BrdU is used as a mutagen; it also inhibits condensation of metaphase chromosomes during cell division.

As used herein, ribosomal RNA (rRNA) is the specialized RNA that forms part of the structure of a ribosome and participates in the synthesis of proteins. Ribosomal RNA is produced by transcription of genes which, in eukaryotic cells, are present in multiple copies. In human cells, the approximately 250 copies of rRNA genes (i.e., genes which encode rRNA) per haploid genome are spread out in clusters on at least five different chromosomes (chromosomes 13, 14, 15, 21 and 22). In mouse cells, the presence of ribosomal DNA (rDNA, which is DNA containing sequences that encode rRNA) has been verified on at least 11 pairs out of 20 mouse chromosomes (chromosomes 5, 6, 7, 9, 11, 12, 15, 16, 17, 18, and 19)

(see e.g., Rowe *et al.* (1996) *Mamm. Genome 7*:886-889 and Johnson *et al.* (1993) *Mamm. Genome 4*:49-52). In *Arabidopsis thaliana* the presence of rDNA has been verified on chromosomes 2 and 4 (18S, 5.8S, and 25S rDNA) and on chromosomes 3,4, and 5 (5S rDNA)(see The Arabidopsis Genome Initiative (2000) Nature 408:796-815). In eukaryotic cells, the multiple copies of the highly conserved rRNA genes are located in a tandemly arranged series of rDNA units, which are generally about 40-45 kb in length and contain a transcribed region and a nontranscribed region known as spacer (i.e., intergenic spacer) DNA which can vary in length and sequence. In the human and mouse, these tandem arrays of rDNA units are located adjacent to the pericentric satellite DNA sequences (heterochromatin). The regions of these chromosomes in which the rDNA is located are referred to as nucleolar organizing regions (NOR) which loop into the nucleolus, the site of ribosome production within the cell nucleus.

As used herein, a megachromosome refers to a chromosome that, except for introduced heterologous DNA, is substantially composed of heterochromatin. Megachromosomes are made up of an array of repeated amplicons that contain two inverted megareplicons bordered by introduced heterologous DNA (see, *e.g.*, Figure 3 of U.S. Patent No. 6,077,697 for a schematic drawing of a megachromosome). For purposes herein, a megachromosome is about 50 to 400 Mb, generally about 250-400 Mb. Shorter variants are also referred to as truncated megachromosomes (about 90 to 120 or 150 Mb), dwarf megachromosomes (~150-200 Mb), and a micro-megachromosome (~50-90 Mb, typically 50-60 Mb). For purposes herein, the term

megachromosome refers to the overall repeated structure based on an array of repeated chromosomal segments (amplicons) that contain two inverted megareplicons bordered by any inserted heterologous DNA. The size will be specified.

As used herein, gene therapy involves the transfer or insertion of nucleic acid molecules into certain cells, which are also referred to as target cells, to produce specific products that are involved in preventing, curing, correcting, controlling or modulating diseases, disorders and deleterious conditions. The nucleic acid is introduced into the selected target cells in a manner such that the nucleic acid is expressed and a product encoded thereby is produced. Alternatively, the nucleic acid may in some manner mediate expression of DNA that encodes a therapeutic product. This product may be a therapeutic compound, which is produced in therapeutically effective amounts or at a therapeutically useful time. It may also encode a product, such as a peptide or RNA, that in some manner mediates, directly or indirectly, expression of a therapeutic product. Expression of the nucleic acid by the target cells within an organism afflicted with a disease or disorder thereby provides for modulation of the disease or disorder. The nucleic acid encoding the therapeutic product may be modified prior to introduction into the cells of the afflicted host in order to enhance or otherwise alter the product or expression thereof.

For use in gene therapy, cells can be transfected *in vitro*, followed by introduction of the transfected cells into an organism. This is often referred to as *ex vivo* gene therapy. Alternatively, the cells can be transfected directly *in vivo* within an organism.

As used herein, therapeutic agents include, but are not limited to, growth factors, antibodies, cytokines, such as tumor necrosis factors and interleukins, and cytotoxic agents and other agents disclosed herein and

known to those of skill in the art. Such agents include, but are not limited to, tumor necrosis factor, α-interferon, β-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator; or, biological response modifiers such as, for example, lymphokines, interleukin- I (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), granulocyte macrophage colony stimulating factor (GMCSF), granulocyte colony stimulating factor (G-CSF), erythropoietin (EPO), pro-coagulants such as tissue factor and tissue factor variants, pro-apoptotic agents such FAS-ligand, fibroblast growth factors (FGF), nerve growth factor and other growth factors.

As used herein, a therapeutically effective product is a product that is encoded by heterologous DNA that, upon introduction of the DNA into a host, a product is expressed that effectively ameliorates or eliminates the symptoms, manifestations of an inherited or acquired disease or that cures the disease.

As used herein, transgenic plants and animals refer to plants and animals in which heterologous or foreign nucleic acid is expressed or in which the expression of a gene naturally present in the plant or animal has been altered by virtue of introduction of heterologous or foreign nucleic acid.

As used herein, IRES (internal ribosome entry site; see, *e.g.*, SEQ ID No. 27 and nucleotides 2736-3308 SEQ ID No. 28) refers to a region of a nucleic acid molecule, such as an mRNA molecule, that allows internal ribosome entry sufficient to initiate translation, which initiation can be detected in an assay for cap-independent translation (see, *e.g.*, U.S. Patent No. 6,171,821). The presence of an IRES within an mRNA molecule allows cap-independent translation of a linked protein-encoding sequence that otherwise would not be translated.

Internal ribosome entry site (IRES) elements were first identified in picornaviruses, which elements are considered the paradigm for cap-independent translation. The 5′ UTRs of all picornaviruses are long and mediate translational initiation by directly recruiting and binding ribosomes, thereby circumventing the initial cap-binding step. IRES elements are frequently found in viral mRNA, they are rare in non-viral mRNA. Among non-viral mRNA molecules that contain functional IRES elements in their respective 5′ UTRs are those encoding immunoglobulin heavy chain binding protein (BiP) (Macejak *et al.* (1991) *Nature* *353*:90-94); Drosophila Antennapedia (Oh *et al.* (1992) *Genes Dev,* *6*:1643-1653); D. Ultrabithorax (Ye *et al.* (1997) *Mol. Cell Biol.* *17*:1714-21); fibroblast growth factor 2 (Vagner *et al.* (1995) *Mol. Cell Biol.* *15*:35-44); initiation factor eIF4G (Gan *et al.* (1998) *J. Biol. Chem.* *273*:5006-5012); proto-oncogene c-myc (Nanbru *et al.* (1995) *J. Biol. Chem.* *272*:32061-32066; Stoneley (1998) *Oncogene* *16*:423-428); $IRES_H$; from the 5′UTR of NRF1 gene (Oumard et al. (2000) *Mol. and Cell Biol.*, 20(8):2755-2759); and vascular endothelial growth factor (VEGF) (Stein *et al.* (1998) *Mol. Cell Biol.* *18*:3112-9).

As used herein, a promoter, with respect to a region of DNA, refers to a sequence of DNA that contains a sequence of bases that signals RNA polymerase to associate with the DNA and initiate transcription of RNA (such as pol II for mRNA) from a template strand of the DNA. A promoter thus generally regulates transcription of DNA into mRNA. A particular promoter provided herein is the Ferritin heavy chain promoter (excluding the Iron Response Element, located in the 5′UTR), which was joined to the 37bp Fer-1 enhancer element. This promoter is set forth as SEQ ID NO:128. The endogenous Fer-1 enhancer element is located upstream of the Fer-1 promotér (e.g., a Fer-1 oligo was cloned proximal to the core promoter).

As used herein, isolated, substantially pure nucleic acid, such as, for example, DNA, refers to nucleic acid fragments purified according to standard techniques employed by those skilled in the art, such as that found in Sambrook *et al.* ((2001) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 3rd edition).

As used herein, expression refers to the transcription and/or translation of nucleic acid. For example, expression can be the transcription of a gene that may be transcribed into an RNA molecule, such as a messenger RNA (mRNA) molecule. Expression may further include translation of an RNA molecule and translated into peptides, polypeptides, or proteins. If the nucleic acid is derived from genomic DNA, expression may, if an appropriate eukaryotic host cell or organism is selected, include splicing of the mRNA. With respect to an antisense construct, expression may refer to the transcription of the antisense DNA.

As used herein, vector or plasmid refers to discrete elements that are used to introduce heterologous nucleic acids into cells for either expression of the heterologous nucleic acid or for replication of the heterologous nucleic acid. Selection and use of such vectors and plasmids are well within the level of skill of the art.

As used herein, transformation/transfection refers to the process by which nucleic acid is introduced into cells. The terms transfection and transformation refer to the taking up of exogenous nucleic acid, e.g., an expression vector, by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, by *Agrobacterium*-mediated transformation, protoplast transformation (including polyethylene glycol (PEG)-mediated transformation, electroporation, protoplast fusion, and microcell fusion), lipid-mediated delivery, liposomes, electroporation,

sonoporation, microinjection, particle bombardment and silicon carbide whisker-mediated transformation and combinations thereof (see, *e.g.*, Paszkowski *et al.* (1984) *EMBO J. 3*:2717-2722; Potrykus *et al.* (1985) *Mol. Gen. Genet. 199*:169-177; Reich *et al.* (1986) *Biotechnology 4*:1001-1004; Klein *et al.* (1987) *Nature 327*:70-73; U.S. Patent No. 6,143,949; Paszkowski *et al.* (1989) *in Cell Culture and Somatic Cell Genetics of Plants, Vol. 6*, Molecular Biology of Plant Nuclear Genes, eds. Schell, J and Vasil, L.K. Academic Publishers, San Diego, California, p. 52-68; and Frame *et al.* (1994) *Plant J. 6*:941-948), direct uptake using calcium phosphate (CaPO4; see,*e.g.*, Wigler *et al.* (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*:1373-1376), polyethylene glycol (PEG)-mediated DNA uptake, lipofection (see, *e.g.*, Strauss (1996) *Meth. Mol. Biol. 54*:307-327), microcell fusion (see, EXAMPLES, see, also Lambert (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*:5907-5911; U.S. Patent No. 5,396,767, Sawford *et al.* (1987) *Somatic Cell Mol. Genet. 13*:279-284; Dhar *et al.* (1984) *Somatic Cell Mol. Genet. 10*:547-559; and McNeill-Killary *et al.* (1995) *Meth. Enzymol. 254*:133-152), lipid-mediated carrier systems (see, e.g., Teifel *et al.* (1995) *Biotechniques 19*:79-80; Albrecht *et al.* (1996) *Ann. Hematol. 72*:73-79; Holmen *et al.* (1995) *In Vitro Cell Dev. Biol. Anim. 31*:347-351; Remy *et al.* (1994) *Bioconjug. Chem. 5*:647-654; Le Bolch *et al.* (1995) *Tetrahedron Lett. 36*:6681-6684; Loeffler *et al.* (1993) *Meth. Enzymol. 217*:599-618) or other suitable method. Methods for delivery of *ACes* are described in copending U.S. application Serial No. 09/815,979. Successful transfection is generally recognized by detection of the presence of the heterologous nucleic acid within the transfected cell, such as, for example, any visualization of the heterologous nucleic acid or any indication of the operation of a vector within the host cell.

As used herein, "delivery," which is used interchangeably with "transfection," refers to the process by which exogenous nucleic acid molecules are transferred into a cell such that they are located inside the cell. Delivery of nucleic acids is a distinct process from expression of nucleic acids.

As used herein, injected refers to the microinjection, such as by use of a small syringe, needle, or pipette, for injection of nucleic acid into a cell.

As used herein, substantially homologous DNA refers to DNA that includes a sequence of nucleotides that is sufficiently similar to another such sequence to form stable hybrids, with each other or a reference sequence, under specified conditions.

It is well known to those of skill in this art that nucleic acid fragments with different sequences may, under the same conditions, hybridize detectably to the same "target" nucleic acid. Two nucleic acid fragments hybridize detectably, under stringent conditions over a sufficiently long hybridization period, because one fragment contains a segment of at least about 10, 14 or 16 or more nucleotides in a sequence that is complementary (or nearly complementary) to a substantially contiguous sequence of at least one segment in the other nucleic acid fragment. If the time during which hybridization is allowed to occur is held constant, at a value during which, under preselected stringency conditions, two nucleic acid fragments with complementary base-pairing segments hybridize detectably to each other, departures from exact complementarity can be introduced into the base-pairing segments, and base-pairing will nonetheless occur to an extent sufficient to make hybridization detectable. As the departure from complementarity between the base-pairing segments of two nucleic acids becomes larger, and as

conditions of the hybridization become more stringent, the probability decreases that the two segments will hybridize detectably to each other.

Two single-stranded nucleic acid segments have "substantially the same sequence", if (a) both form a base-paired duplex with the same segment, and (b) the melting temperatures of the two duplexes in a solution of 0.5 X SSPE differ by less than 10°C. If the segments being compared have the same number of bases, then to have "substantially the same sequence", they will typically differ in their sequences at fewer than 1 base in 10. Methods for determining melting temperatures of nucleic acid duplexes are well known (see, *e.g.*, Meinkoth *et al.* (1984) *Anal. Biochem.* *138*:267-284 and references cited therein).

As used herein, a nucleic acid probe is a DNA or RNA fragment that includes a sufficient number of nucleotides to specifically hybridize to DNA or RNA that includes complementary or substantially complementary sequences of nucleotides. A probe may contain any number of nucleotides, from as few as about 10 and as many as hundreds of thousands of nucleotides. The conditions and protocols for such hybridization reactions are well known to those of skill in the art as are the effects of probe size, temperature, degree of mismatch, salt concentration and other parameters on the hybridization reaction. For example, the lower the temperature and higher the salt concentration at which the hybridization reaction is carried out, the greater the degree of mismatch that may be present in the hybrid molecules.

To be used as a hybridization probe, the nucleic acid is generally rendered detectable by labeling it with a detectable moiety or label, such as ^{32}P, ^{3}H and ^{14}C, or by other means, including chemical labeling, such as by nick-translation in the presence of deoxyuridylate biotinylated at the 5'-position of the uracil moiety. The resulting probe includes the biotinylated uridylate in place of thymidylate residues and can be detected

(via the biotin moieties) by any of a number of commercially available detection systems based on binding of streptavidin to the biotin. Such commercially available detection systems can be obtained, for example, from Enzo Biochemicals, Inc. (New York, NY). Any other label known to those of skill in the art, including non-radioactive labels, may be used as long as it renders the probes sufficiently detectable, which is a function of the sensitivity of the assay, the time available (for culturing cells, extracting DNA, and hybridization assays), the quantity of DNA or RNA available as a source of the probe, the particular label and the means used to detect the label.

Once sequences with a sufficiently high degree of homology to the probe are identified, they can readily be isolated by standard techniques (see, *e.g.*, Sambrook *et al.* (2001) *Molecular Cloning: A Laboratory Manual*, 3rd Edition, Cold Spring Harbor Laboratory Press).

As used herein, conditions under which DNA molecules form stable hybrids are considered substantially homologous, and a DNA or nucleic acid homolog refers to a nucleic acid that includes a preselected conserved nucleotide sequence, such as a sequence encoding a polypeptide. By the term "substantially homologous" is meant having at least 75%, preferably 80%, preferably at least 90%, most preferably at least 95% homology therewith or a less percentage of homology or identity and conserved biological activity or function.

The terms "homology" and "identity" are often used interchangeably. In this regard, percent homology or identity may be determined, for example, by comparing sequence information using a GAP computer program. The GAP program utilizes the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443 (1970), as revised by Smith and Waterman (*Adv. Appl. Math.* 2:482 (1981). Briefly, the GAP program defines similarity as the number of aligned symbols (i.e.,

nucleotides or amino acids) which are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program may include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.* 14:6745 (1986), as described by Schwartz and Dayhoff, eds., *ATLAS OF PROTEIN SEQUENCE AND STRUCTURE*, National Biomedical Research Foundation, pp. 353-358 (1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

By sequence identity, the number of conserved amino acids are determined by standard alignment algorithms programs, and are used with default gap penalties established by each supplier. Substantially homologous nucleic acid molecules would hybridize typically at moderate stringency or at high stringency all along the length of the nucleic acid of interest. Preferably the two molecules will hybridize under conditions of high stringency. Also contemplated are nucleic acid molecules that contain degenerate codons in place of codons in the hybridizing nucleic acid molecule.

Whether any two nucleic acid molecules have nucleotide sequences that are at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% "identical" can be determined using known computer algorithms such as the "FAST A" program, using for example, the default parameters as in Pearson and Lipman, *Proc. Natl. Acad. Sci. USA 85*:2444 (1988). Alternatively the BLAST function of the National Center for Biotechnology Information database may be used to determine relative sequence identity.

In general, sequences are aligned so that the highest order match is obtained. "Identity" *per se* has an art-recognized meaning and can be

calculated using published techniques. (See, *e.g.*: *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). While there exist a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to skilled artisans (Carillo, H. & Lipton, D., *SIAM J Applied Math 48*:1073 (1988)). Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in Guide to Huge Computers, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo, H. & Lipton, D., *SIAM J Applied Math 48*:1073 (1988). Methods to determine identity and similarity are codified in computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCG program package (Devereux, J., *et al., Nucleic Acids Research 12(I)*:387 (1984)), BLASTP, BLASTN, FASTA (Atschul, S.F., *et al., J Molec Biol 215*:403 (1990)).

Therefore, as used herein, the term "identity" represents a comparison between a test and a reference polypeptide or polynucleotide. For example, a test polypeptide may be defined as any polypeptide that is 90% or more identical to a reference polypeptide.

As used herein, the term at least "90% identical to" refers to percent identities from 90 to 99.99 relative to the reference polypeptides. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polynucleotide length of

100 amino acids are compared. No more than 10% (i.e., 10 out of 100) amino acids in the test polypeptide differs from that of the reference polypeptides. Similar comparisons may be made between a test and reference polynucleotides. Such differences may be represented as point mutations randomly distributed over the entire length of an amino acid sequence or they may be clustered in one or more locations of varying length up to the maximum allowable, e.g. 10/100 amino acid difference (approximately 90% identity). Differences are defined as nucleic acid or amino acid substitutions, or deletions.

As used herein: stringency of hybridization in determining percentage mismatch encompass the following conditions or equivalent conditions thereto:

1) high stringency: 0.1 x SSPE or SSC, 0.1% SDS, 65°C
2) medium stringency: 0.2 x SSPE or SSC, 0.1% SDS, 50°C
3) low stringency: 1.0 x SSPE or SSC, 0.1% SDS, 50°C

or any combination of salt and temperature and other reagents that result in selection of the same degree of mismatch or matching. Equivalent conditions refer to conditions that select for substantially the same percentage of mismatch in the resulting hybrids. Additions of ingredients, such as formamide, Ficoll, and Denhardt's solution affect parameters such as the temperature under which the hybridization should be conducted and the rate of the reaction. Thus, hybridization in 5 X SSC, in 20% formamide at 42° C is substantially the same as the conditions recited above hybridization under conditions of low stringency. The recipes for SSPE, SSC and Denhardt's and the preparation of deionized formamide are described, for example, in Sambrook *et al.* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Chapter 8; see, Sambrook *et al.*, vol. 3, p. B.13, see, also, numerous catalogs that describe commonly used laboratory solutions. It is understood that

equivalent stringencies may be achieved using alternative buffers, salts and temperatures. As used herein, all assays and procedures, such as hybridization reactions and antibody-antigen reactions, unless otherwise specified, are conducted under conditions recognized by those of skill in the art as standard conditions.

As used herein, conservative amino acid substitutions, such as those set forth in Table 1, are those that do not eliminate biological activity. Suitable conservative substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, *e.g.*, Watson *et al. Molecular Biology of the Gene*, 4th Edition, 1987, The Bejacmin/Cummings Pub. co., p.224). Conservative amino acid substitutions are made, for example, in accordance with those set forth in TABLE 1 as follows:

TABLE 1

Original residue	Conservative substitution
Ala (A)	Gly; Ser, Abu
Arg (R)	Lys, orn
Asn (N)	Gln; His
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
Gly (G)	Ala; Pro
His (H)	Asn; Gln
Ile (I)	Leu; Val; Met; Nle; Nva
Leu (L)	Ile; Val; Met; Nle; Nva
Lys (K)	Arg; Gln; Glu
Met (M)	Leu; Tyr; Ile; NLe Val
Ornithine	Lys; Arg
Phe (F)	Met; Leu; Tyr
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp; Phe
Val (V)	Ile; Leu; Met; Nle; Nva

Other substitutions are also permissible and may be determined empirically or in accord with known conservative substitutions.

As used herein, the amino acids, which occur in the various amino acid sequences appearing herein, are identified according to their well-known, three-letter or one-letter abbreviations. The nucleotides, which occur in the various DNA fragments, are designated with the standard single-letter designations used routinely in the art.

As used herein, a splice variant refers to a variant produced by differential processing of a primary transcript of genomic DNA that results in more than one type of mRNA.

As used herein, a probe or primer based on a nucleotide sequence includes at least 10, 14, 16, 30 or 100 contiguous nucleotides from the reference nucleic acid molecule.

As used herein, recombinant production by using recombinant DNA methods refers to the use of the well known methods of molecular biology for expressing proteins encoded by cloned DNA.

As used herein, biological activity refers to the *in vivo* activities of a compound or physiological responses that result upon *in vivo* administration of a compound, composition or other mixture. Biological activity, thus, encompasses therapeutic effects and pharmaceutical activity of such compounds, compositions and mixtures. Biological activities may be observed in *in vitro* systems designed to test or use such activities. Thus, for purposes herein the biological activity of a luciferase is its oxygenase activity whereby, upon oxidation of a substrate, light is produced.

The terms substantially identical or similar varies with the context as understood by those skilled in the relevant art and generally means at least 40, 60, 80, 90, 95 or 98%.

As used herein, substantially identical to a product means sufficiently similar so that the property is sufficiently unchanged so that the substantially identical product can be used in place of the product.

As used herein, substantially pure means sufficiently homogeneous to appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound may, however, be a mixture of stereoisomers or isomers. In such instances, further purification might increase the specific activity of the compound.

As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. The vectors typically remain episomal, but may be designed to effect integration of a gene or portion thereof into a chromosome of the genome. Also contemplated are vectors that are artificial chromosomes, such as yeast artificial chromosomes and mammalian artificial chromosomes. Selection and use of such vehicles are well known to those of skill in the art. An expression vector includes vectors capable of expressing DNA that is operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector

refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

As used herein, protein-binding-sequence refers to a protein or peptide sequence that is capable of specific binding to other protein or peptide sequences generally, to a set of protein or peptide sequences or to a particular protein or peptide sequence.

As used herein, a composition refers to any mixture of two or more ingredients. It may be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous or any combination thereof.

As used herein, a combination refers to any association between two or more items.

As used herein, fluid refers to any composition that can flow. Fluids thus encompass compositions that are in the form of semi-solids, pastes, solutions, aqueous mixtures, gels, lotions, creams and other such compositions.

As used herein, a cellular extract refers to a preparation or fraction that is made from a lysed or disrupted cell.

As used herein, the term "subject" refers to animals, plants, insects, and birds and other phyla, genera and species into which nucleic acid molecules may be introduced. Included are higher organisms, such as mammals, fish, insects and birds, including humans, primates, cattle, pigs, rabbits, goats, sheep, mice, rats, guinea pigs, hamsters, cats, dogs, horses, chicken and others.

As used herein, flow cytometry refers to processes that use a laser based instrument capable of analyzing and sorting out cells and or chromosomes based on size and fluorescence.

As used herein, the abbreviations for any protective groups, amino acids and other compounds, are, unless indicated otherwise, in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature (see, (1972) *Biochem.* *11*:942-944).

B. Recombination systems

Site-specific recombination systems typically contain three elements: a pair of DNA sequences (the site-specific recombination sequences) and a specific enzyme (the site-specific recombinase). The site-specific recombinase catalyzes a recombination reaction between two site-specific recombination sequences.

A number of different site-specific recombinase systems are available and/or known to those of skill in the art, including, but not limited to: the Cre/*lox* recombination system using CRE recombinase (see, *e.g.*, SEQ ID Nos. 58 and 59) from the Escherichia coli phage P1 (see, *e.g.*, Sauer (1993) *Methods in Enzymology 225*:890-900; Sauer *et al.* (1990) *The New Biologist* 2:441-449), Sauer (1994) *Current Opinion in Biotechnology 5*:521-527; Odell *et al.* (1990) *Mol Gen Genet. 223*:369-378; Lasko *et al.* (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*:6232-6236; U.S. Patent No. 5,658,772), the FLP/FRT system of yeast using the FLP recombinase (see, SEQ ID Nos. 60 and 61) from the 2μ episome of Saccharomyces cerevisiae (Cox (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*:4223; Falco *et al.* (1982) *Cell 29*:573-584; Golic *et al.* (1989) *Cell*59:499-509; U.S. Patent No. 5,744,336), the resolvases, including Gin recombinase of phage Mu (Maeser *et al.* (1991) *Mol Gen Genet. 230*:170-176; Klippel, A. et al (1993) *EMBO J. 12*:1047-1057; see, *e.g.*,

SEQ ID Nos. 64-67), Cin, Hin, $\alpha\delta$ Tn3; the Pin recombinase of *E. coli* (see, *e.g.*, SEQ ID Nos. 68 and 69; Enomoto *et al.* (1983) *J Bacteriol.* *6*:663-668), the R/RS system of the pSR1 plasmid of Zygosaccharomyces rouxii (Araki *et al.* (1992) *J. Mol. Biol.* 225:25-37; Matsuzaki *et al.* (1990) *J. Bacteriol. 172*: 610-618) and site-specific recombinases from Kluyveromyces drosophilarium (Chen *et al.* (1986) *Nucleic Acids Res. 314*:4471-4481) and Kluyveromyces waltii (Chen *et al.* (1992) *J. Gen. Microbiol. 138*:337-345). Other systems are known to those of skill in the art (Stark *et al. Trends Genet. 8*:432-439; Utatsu *et al.* (1987) *J. Bacteriol. 169*:5537-5545; see, also, U.S. Patent No. 6,171,861).

Members of the highly related family of site-specific recombinases, the resolvase family, such as $\gamma\delta$, Tn3 resolvase, Hin, Gin, and Cin are also available. Members of this family of recombinases are typically constrained to intramolecular reactions (e.g., inversions and excisions) and can require host-encoded factors. Mutants have been isolated that relieve some of the requirements for host factors (Maeser *et al.* (1991) *Mol. Gen. Genet. 230*:170-176), as well as some of the constraints of intramolecular recombination (see, U.S. Patent No. 6,171,861).

The bacteriophage P1 Cre/lox and the yeast FLP/FRT systems are particularly useful systems for site-specific integration, inversion or excision of heterologous nucleic acid into, and out of, chromosomes, particularly *ACes* as provided herein. In these systems a recombinase (Cre or FLP) interacts specifically with its respective site-specific recombination sequence (lox or FRT, respectively) to invert or excise the intervening sequences. The sequence for each of these two systems is relatively short (34 bp for lox and 47 bp for FRT).

The FLP/FRT recombinase system has been demonstrated to function efficiently in plant cells (U.S. Patent No. 5,744,386), and, thus, can be used for producing plant artificial chromosome platforms. In

general, short incomplete FRT sites leads to higher accumulation of excision products than the complete full-length FRT sites. The system catalyzes intra- and intermolecular reactions, and, thus, can be used for DNA excision and integration reactions. The recombination reaction is reversible and this reversibility can compromise the efficiency of the reaction in each direction. Altering the structure of the site-specific recombination sequences is one approach to remedying this situation. The site-specific recombination sequence can be mutated in a manner that the product of the recombination reaction is no longer recognized as a substrate for the reverse reaction, thereby stabilizing the integration or excision event.

In the Cre-lox system, discovered in bacteriophage P1, recombination between loxP sites occurs in the presence of the Cre recombinase (see, *e.g.*, U.S. Patent No. 5,658,772). This system can be used to insert, invert or excise nucleic acid located between two lox sites. Cre can be expressed from a vector. Since the lox site is an asymmetrical nucleotide sequence, lox sites on the same DNA molecule can have the same or opposite orientation with respect to each other. Recombination between lox sites in the same orientation results in a deletion of the DNA segment located between the two lox sites and a connection between the resulting ends of the original DNA molecule. The deleted DNA segment forms a circular molecule of DNA. The original DNA molecule and the resulting circular molecule each contain a single lox site. Recombination between lox sites in opposite orientations on the same DNA molecule result in an inversion of the nucleotide sequence of the DNA segment located between the two lox sites. In addition, reciprocal exchange of DNA segments proximate to lox sites located on two different DNA molecules can occur. All of these recombination events are catalyzed by the product of the Cre coding region.

Any site-specific recombinase system known to those of skill in the art is contemplated for use herein. It is contemplated that one or a plurality of sites that direct the recombination by the recombinase are introduced into an artificial chromosome to produce platform *ACes*. The resulting platform *ACes* are introduced into cells with nucleic acid encoding the cognate recombinase, typically on a vector, and nucleic acid encoding heterologous nucleic acid of interest linked to the appropriate recombination site for insertion into the platform *ACes*. The recombinase-encoding-nucleic acid may be introduced into the cells on the same vector, or a different vector, encoding the heterologous nucleic acid.

An *E. coli* phage lambda integrase system for *ACes* platform engineering and for artificial chromosome engineering is provided (Lorbach *et al.* (2000) *J. Mol. Biol 296*:1175-1181). The phage lambda integrase (Landy, A. (1989) *Annu. Rev. Biochem. 58*:913-94) is adapted herein and the cognate *att* sites are provided. Chromosomes, including *ACes*, engineered to contain one or a plurality of *att* sites are provided, as are vectors encoding a mutant integrase that functions in the absence other factors. Methods using the modified chromosomes and vectors for introduction of heterologous nucleic acid are also provided.

For purposes herein, one or more of the sites (e.g., a single site or a pair of sites) required for recombination are introduced into an artificial chromosome, such as an *ACes* chromosome. The enzyme for catalyzing site-directed recombination is introduced with the DNA of interest, or separately, or is engineered onto the artificial chromosome under the control of a regulatable promoter.

As described herein, artificial chromosome platforms containing one or multiple recombination sites are provided. The methods and resulting products are exemplified with the lambda phage Att/Int system, but

similar methods may be used for production of *ACes* platforms with other recombination systems.

The Att/Int system and vectors provided herein are not only intended for engineering *ACes* platforms, but may be used to engineer an Att/Int system into any chromosome. Introduction of att sites into a chromosome will permit engineering of natural chromosomes, such as by permitting targeted integration genes or regulatory regions, and by controlled excision of selected regions. For example, genes encoding a particular trait may be added to a chromosome, such as plant chromosome engineered to contain one or plurality of *att* sites. Such chromosomes may be used for screening DNA to identify genes. Large pieces of DNA can be introduced into cells and the cells screened phenotypically to select those having the desired trait.

C. Platforms

Provided herein are platform artificial chromosomes (platform *ACes*) containing single or multiple site-specific recombination sites. Chromosome-based platform technology permits efficient and tractable engineering and subsequent expression of multiple gene targets. Methods are provided that use DNA vectors and fragments to create platform artificial chromosomes, including animal, particularly mammalian, artificial chromosomes, and plant artificial chromosomes. The artificial chromosomes contain either single or multiple sequence-specific recombination sites suitable for the placement of target gene expression vectors onto the platform chromosome. The engineered chromosome-based platform *ACes* technology is applicable for methods, including cellular and transgenic protein production, transgenic plant and animal production and gene therapy. The platform *ACes* are also useful for producing a library of *ACes* comprising random portions of a given genome (e.g., a mammalian, plant or prokaryotic genome) for genomic

screening; as well as a library of cells comprising different and/or mutually exclusive *ACes* therein.

Exemplary of artificial chromosome platforms are those based on *ACes*. *ACes* artificial chromosomes are non-viral, self-replicating nucleic acid molecules that function as a natural chromosome, having all the elements required for normal chromosomal replication and maintenance within the cell nucleus. *ACes* artificial chromosomes do not rely on integration into the genome of the cell to be effective, and they are not limited by DNA carrying capacity and as such the therapeutic gene(s) of interest, including regulatory sequences, can be engineered into the *ACes*. In addition, *ACes* are stable *in vitro* and *in vivo* and can provide predictable long-term gene expression. Once engineered and delivered to the appropriate cell or embryo, *ACes* work independently alongside host chromosomes, for *ACes* that are predominantly heterochromatin producing only the products (proteins) from the genes it carries. As provided herein *ACes* are modified by introduction of recombination site(s) to provide a platform for ready introduction of heterologous nucleic acid. The *ACes* platforms can be used for production of transgenic animals and plants; as vectors for genetic therapy; for use as protein production systems; for animal models to identify and target new therapeutics; in cell culture for the development and production of therapeutic proteins; and for a variety of other applications.

1. Generation of artificial chromosomes

Artificial chromosomes may be generated by any method known to those of skill in the art. Of particular interest herein are the *ACes* artificial chromosomes, which contain a repeated unit. Methods for production of *ACes* are described in detail in U.S. Patent Nos. 6,025,155 and 6,077,697, which, as with all patents, applications, publications and other disclosure, are incorporated herein in their entirety.

Generation of *de novo ACes*.

ACes can be generated by cotransfecting exogenous DNA—such as a mammary tissue-specific DNA cassette including the gene sequences for a therapeutic protein, with a rDNA fragment and a drug resistance marker gene into the desired eukaryotic cell, such as plant or animal cells, such as murine cells *in vitro*. DNA with a selectable or detectable marker is introduced, and can be allowed to integrate randomly into pericentric heterochromatin or can be targeted to pericentric heterochromatin, such as that in rDNA gene arrays that reside on acrocentric chromosomes, such as the short arms of acrocentric chromosomes. This integration event activates the "megareplicator" sequence and amplifies the pericentric heterochromatin and the exogenous DNA, and duplicates a centromere. Ensuing breakage of this "dicentric" chromosome can result in the production of daughter cells that contain the substantially-original chromosome and the new artificial chromosome. The resulting *ACes* contain all the essential elements needed for stability and replication in dividing cells—centromere, origins of replications, and telomeres. *ACes* have been produced that express marker genes (lacZ, green fluorescent protein, neomycin-resistance, puromycin-resistance, hygromycin-resistance) and genes of interest. Isolated *ACes*, for example, have been successfully transferred intact to rodent, human, and bovine cells by electroporation, sonoporation, microinjection, and transfection with lipids and dendrimers.

To render the creation of *ACes* with desired genes more tractable and efficient, "platform" *ACes* (platform-*ACes*) can be produced that contain defined DNA sequences for enzyme-mediated homologous DNA recombination, such as by Cre or FLP recombinases (Bouhassira *et al.* (1996) *Blood 88(supplement 1)*:190a; Bouhassira *et al.* (1997) *Blood, 90*:3332-3344; Siebler *et al.* (1997) *Biochemistry: 36*:1740-1747;

Siebler *et al.* (1998) *Biochemistry 37*: 6229-6234; and Bethke *et al.* (1997) *Nucl. Acids Res. 25*:2828-2834), and as exemplified herein the lambda phage integrase. A *lox* site contains two 13 bp inverted repeats to which Cre-recombinase binds and an intervening 8 bp core region. Only pairs of sites having identity in the central 6 bp of the core region are proficient for recombination; sites having non-identical core sequences (heterospecific *lox* sites) do not efficiently recombine with each other (Hoess *et al.* (1986) *Nucleic Acids Res. 14*:2287-2300).

Generating acrocentric chromosomes for plant artificial chromosome formation.

In human and mouse cells *de novo* formation of a satellite DNA based artificial chromosome (SATAC, also referred to as *ACes*) can occur in an acrocentric chromosome where the short arm contains only pericentric heterochromatin, the rDNA array, and telomere sequences. Plant species may not have any acrocentric chromosomes with the same physical structure described, but "megareplicator" DNA sequences reside in the plant rDNA arrays, also known as the nucleolar organizing regions (NOR). A structure like those seen in acrocentric mammalian chromosomes can be generated using site-specific recombination between appropriate arms of plant chromosomes.

Approach

Qin *et al.* ((1994) *Proc. Natl. Acad. Sci. U.S.A. 91*:1706-1710, 1994) describes crossing two *Nicotiana tabacum* transgenic plants. One plant contains a construct encoding a promoterless hygromycin-resistance gene preceded by a *lox* site (*lox-hpt*), the other plant carries a construct containing a cauliflower mosaic virus 35S promoter linked to a *lox* sequence and the *cre* DNA recombinase coding region (35S-*lox-cre*). The constructs were introduced separately by infecting leaf explants with *agrobacterium tumefaciens* which carries the kanamycin-resistance gene

(Kan^R). The resultant Kan^R transgenic plants were crossed. Plants that carried the appropriate DNA recombination event were identified by hygromycin-resistance.

Modification of the above for generation of *ACes*

The Kan^R cultivars are initially screened, such as by FISH, to identify two sets of candidate transgenic plants. One set has one construct integrated in regions adjacent to the pericentric heterochromatin on the short arm of any chromosome. The second set of candidate plants has the other construct integrated in the NOR region of appropriate chromosomes. To obtain reciprocal translocation both sites must be in the same orientation. Therefore a series of crosses are required, Kan^R plants generated, and FISH analyses performed to identify the appropriate "acrocentric" plant chromosome for *de novo* plant *ACes* formation.

2. Bacteriophage lambda integrase-based site-specific recombination system

An integral part of the platform technology includes a site-specific recombination system that allows the placement of selected gene targets or genomic fragments onto the platform chromosomes. Any such system may be used. In particular, a method is provided for insertion of additional DNA fragments into the platform chromosome residing in the cell via sequence-specific recombination using the recombinase activity of the bacteriophage lambda integrase. The lambda integrase system is exemplary of the recombination systems contemplated for *ACes*. Any known recombination system, including any described herein, particularly any that operates without the need for additional factors or that, by virtue of mutation, does not require additional factors, is contemplated.

As noted the lambda integrase system provided herein can be used with natural chromosomes and artificial chromosomes in addition to *ACes*. Single or a plurality of recombination sites, which may be the same or different, are introduced into artificial chromosomes to produce artificial chromosome platforms.

3. Creation of bacteriophage lambda integrase site-specific recombination system

The lambda phage-encoded integrase (designated Int) is a prototypical member of the integrase family. Int effects integration and excision of the phage in and out of the *E. coli* genome via recombination between pairs of attachment sites designated *att*B/*att*P and *att*L/*att*R. Each *att* site contains two inverted 9 base pair core Int binding sites and a 7 base pair overlap region that is identical in wild-type *att* sites. Each site, except for *att*B contains additional Int binding sites. In flanking regions, there are recognition sequences for accessory DNA binding proteins, such as integration host factor (IHF), factor for inversion stimulation (FIS) and the phage encoded excision protein (XIS). Except for *att*B, Int is a heterobivalent DNA-binding protein and, with assistance from the accessory proteins and negative DNA supercoiling, binds simultaneously to core and arm sites within the same *att* site.

Int, like Cre and FLP, executes an ordered sequential pair of strand exchanges during integrative and excisive recombination. The natural pairs of target sequences for Int, *att*B and *att*P or *att*L and *att*R are located on the same or different DNA molecules resulting in intra or intermolecular recombination, respectively. For example, intramolecular recombination occurs between inversely oriented *att*B and *att*P, or between *att*L and *att*R sequences, respectively, leading to inversion of the intervening DNA segment.

Like the recombinase systems, such as Cre and FLP, Int directs site-specific recombination. Unlike the other systems, such Cre and FLP, Int generally requires additional protein factors for integrative and excisive recombination and negative supercoiling for integrative recombination. Hence, the Int system had not been used in eukaryotic targeting systems.

Mutant Int proteins, designated Int-h (E174K) and a derivative thereof Int-h/218(E174K/E218K) do not require accessory proteins to perform intramolecular integrative and excisive recombination in co-transfection assays in human cells (Lorbach *et al.* (2000) *J Mol. Biol.* *296*:1175-1181); wild-type Int does not catalyze intramolecular recombination in human cells harboring target sites *att*B and *att*P. Hence it had been demonstrated that mutant Int can catalyze factor-independent recombination events in human cells.

There has been no demonstration by others that this system can be used for engineering of eukaryotic genomes or chromosomes. Provided herein are chromosomes, including artificial chromosomes, such as but not limited to *ACes* that contain *att* sites (e.g., platform *ACes*), and the use of such chromosomes for targeted integration of heterologous DNA into such chromosomes in eukaryotic cells, including animal, such as rodent and human, and plant cells. Mutant Int provided herein is shown to effect site-directed recombination between sites in artificial chromosomes and vectors containing cognate sites.

An additional component of the chromosome-based platform technology is the site-specific integration of target DNA sequences onto the platform. For this the native bacteriophage lambda integrase has been modified to carry out this sequence specific DNA recombination event in eukaryotic cells. The bacteriophage lambda integrase and its cognate DNA substrate att is a member of the site-specific recombinase family that also includes the bacteriophage P1 Cre/lox system as well as

the *Saccharomyces cerevisiae* 2 micron based FLP/FRT system (see, *e.g.*, Landy (1989) *Ann. Rev. Biochem 58*:913-949; Hoess *et al.* (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*:3398-3402; Broach *et al.* (1982) *Cell 29*:227-234).

By combining DNA endonuclease and DNA ligase activity these recombinases recognize and catalyze DNA exchanges between sequences flanking the recognition site. During the integration of lambda genome into the *E. coli* (lambda recombination) genome, the phage integrase (INT) in association with accessory proteins catalyzes the DNA exchange between the attP site of the phage genome and the attB site of the bacterial genome resulting in the formation of attL and attR sites (Figure 6). The engineered bacteriophage lambda integrase has been produced herein to carry out an intermolecular DNA recombination event between an incoming DNA molecule (primarily on a vector containing the bacterial attB site) and the chromosome-based platform carrying the lambda attP sequence independent of lambda bacteriophage or bacterial accessory proteins.

In contrast to the bi-directional Cre/lox and FLP/FRT system, the engineered lambda recombination system derived for chromosome-based platform technology is advantageously unidirectional because accessory proteins, which are absent, are required for excision of integrated nucleic acid upon further exposure to the lambda Int recombinase.

4. Creation of platform chromosome containing single or multiple sequence-specific recombination sites

a. Multiple sites

For the creation of a platform chromosome containing multiple, sequence-specific recombination sites, artificial chromosomes are produced as depicted in Figure 5 and Example 3. As discussed above, artificial chromosomes can be produced using any suitable methodology,

including those described in U.S. Patent Nos. 5,288,625; 5,712,134; 5,891,691; 6,025,155. Briefly, to prepare artificial chromosomes containing multiple recombination (e.g., integration) sites, nucleic acid (either in the form a one or more plasmids, such as the plasmid pSV40193attPsensePUR set forth in Example 3) is targeted into an amplifiable region of a chromosome, such as the pericentric region of a chromosome. Among such regions are the rDNA gene loci in acrocentric mammalian chromosomes. Hence, targeting nucleic acid for integration into the rDNA region of mammalian acrocentric chromosomes can include the mouse rDNA fragments (for targeting into rodent cell lines) or large human rDNA regions on BAC/PAC vectors (or subclones thereof in standard vectors) for targeting into human acrocentric chromosomes, such as for human gene therapy applications. The targeting nucleic acid generally includes a detectable or selectable marker, such as antibiotic resistance, such as puromycin and hygromycin, a recombination site (such as attP, attB, attL, attR or the like), and/or human selectable markers as required for gene therapy applications. Cells are grown under conditions that result in amplification and ultimately production of *ACes* artificial chromosomes having multiple recombination (e.g.,integration) sites therein. *ACes* having the desired size are selected for further engineering.

b. Creation of platform chromosome containing a single sequence-specific recombination site

In this method a mammalian platform artificial chromosome is generated containing a single sequence-specific recombination site. In the Example below, this approach is demonstrated using a puromycin resistance marker for selection and a mouse rDNA fragment for targeting into the rDNA locus on mouse acrocentric chromosomes. Other selection markers and targeting DNA sequences as desired and known to those of

skill in the art can be used. Additional resistance markers include genes conferring resistance to the antibiotics neomycin, blasticidin, hygromycin and zeocin. For applications, such as gene therapy in which potentially immunogenic responses are to be avoided, host, such as human, derived selectable markers or markers detectable with monoclonal antibodies (MAb) followed by fluorescent activated cell sorting (FACS) can be used. Examples in this class include, but are not limited to: human nerve growth factor receptor (detection with MAb); truncated human growth factor receptor (detection with MAb); mutant human dihydrofolate reductase (DHFR; detectable using a fluorescent methotrexate substrate); secreted alkaline phosphatase (SEAP; detectable with fluorescent substrate); thymidylate synthase (TS; confers resistance to fluorodeoxyuridine); human CAD gene (confers resistance to N-phosphonacetyl-L-aspartate (PALA)).

To construct a platform artificial chromosome with a single site, an *ACes* artificial chromosome (or other artificial chromosome of interest) can be produced containing a selectable marker. A single sequence specific recombination site is targeted onto *ACes* via homologous recombination. For this, DNA sequences containing the site-specific recombination sequence are flanked with DNA sequences homologous to a selected sequence in the chromosome. For example, when using a chromosome containing rDNA or satellite DNA, such DNA can be used as homologous sequences to target the site-specific recombination sequence onto the chromosome. A vector is designed to have these homologous sequences flanking the site-specific recombination site and, after the appropriate restriction enzyme digest to generate free ends of homology to the chromosome, the DNA is transfected into cells harboring the chromosome. After transfection and integration of the site-specific cassette, homologous recombination events onto the platform

chromosome are subcloned and identified, for example by screening single cell subclones via expression of resistance or a fluorescent marker and PCR analysis. In one embodiment, a platform artificial chromosome, such as a platform *ACes*, that contains a single copy of the recombination site is selected. Examples 2B and 2D exemplify the process, and Figure 3 provides a diagram depicting one method for the creation of a platform mammalian chromosome containing a single sequence-specific recombination site.

5. Lambda integrase mediated recombination of target gene expression vector onto platform chromosome

The third component of the chromosome-based platform technology involves the use of target gene expression vectors carrying, for example, genes for gene therapy, genes for transgenic animal or plant production, and those required for cellular protein production of interest. Using lambda integrase mediated site-specific recombination, or any other recombinase-mediated site-specific recombination, the target gene expression vectors are introduced onto the selected chromosome platform. The use of target gene expression vector permits use of the *de novo* generated chromosome-based platforms for a wide range of gene targets. Furthermore, chromosome platforms containing multiple *att*P sites provides the opportunity to incorporate multiple gene targets onto a single platform, thereby providing for expression of multiple gene targets, including the expression of cellular and genetic regulatory genes and the expression of all or parts of metabolic pathways. In addition to expressing small target genes, such as cDNA and hybrid cDNA/artificial intron constructs, the chromosome-based platform can be used for engineering and expressing large genomic fragments carrying target genes along with its endogenous genomic promoter sequences. This is of importance, for example, where the therapy requires precise cell specific

expression and in instances where expression is best achieved from genomic clones rather than cDNA clones. Figure 9 provides a diagram summarizing one embodiment of the chromosome-based technology.

A feature of the target gene expression vector that is of interest to include is a promoterless marker gene, which as exemplified (see, Figure 9) contains an upstream *att*B site (marker 2 on Figure 9). The nucleic acid encoding the marker is not expressed unless it is placed downstream from a promoter sequence. Using the recombinase technology provided herein, such as the lambda integrase technology (λINT_{E174R} on figure 8) provided herein, site-specific recombination between the attB site on the vector and the promoter-attP site (in the "sense" orientation) on the chromosome-based platform results in the expression of marker 2 on the target gene expression vector, thereby providing a positive selection for the lambda INT mediated site-specific recombination event. Site-specific recombination events on the chromosome-based platform versus random integrations next to a promoter in the genome (false positive) can be quickly screened by designing primers to detect the correct event by PCR. Examples of suitable marker 2 genes, include, but are not limited to, genes that confer resistance to toxic compounds or antibiotics, fluorescence activated cell sorting (FACS) sortable cell surface markers and various fluorescent markers. Examples of these genes include, but are not limited to, human L26a^R (human homolog of *Saccharomyces cerevisiae* CYH^8 gene), neomycin, puromycin, blasticidin, CD24 (see, e.g., US Patents 5,804,177 and 6,074,836), truncated CD4, truncated low affinity nerve growth factor receptor (LNGFR), truncated LDL receptor, truncated human growth hormone receptor, GFP, RFP, BFP.

The target gene expression vectors contain a gene (target gene) for expression from the chromosome platform. The target gene can be expressed using various constitutive or regulated promoter systems

across various mammalian species. For the expression of multiple target genes within the same target gene expression vector, the expression of the multiple targets can be coordinately regulated via viral-based or human internal ribosome entry site (IRES) elements (see, *e.g.*, Jackson *et al.* (1990) *Trends Biochem Sci. 15*: 477-83; Oumard *et al.* (2000) *Mol. Cell. Biol. 20*: 2755-2759). Furthermore, using IRES type elements linked to a downstream fluorescent marker, *e.g.*, green, red or blue fluorescent proteins (GFP, RFP, BFP) allows for the identification of high expressing clones from the integrated target gene expression vector.

In certain embodiments described herein, the promoterless marker can be transcriptionally downstream of the heterologous nucleic acid, wherein the heterologous nucleic acid encodes a heterologous protein, and wherein the expression level of the selectable marker is transcriptionally linked to the expression level of the heterologous protein. In addition, the selectable marker and the heterologous nucleic acid can be transcriptionally linked by the presence of a IRES between them. As set forth herein the selectable marker is selected from the group consisting of an antibiotic resistance gene, and a detectable protein, wherein the detectable protein is chromogenic or fluorescent. Expression from the target gene expression vector integrated onto the chromosome-based platform can be further enhanced using genomic insulator/boundary elements. The incorporation of insulator sequences into the target gene expression vector helps define boundaries in chromatin structure and thus minimizes influence of chromatin position effects/gene silencing on the expression of the target gene (Bell *et al.* (1999) *Current Opinion in Genetics and Development 9*:191-198; Emery *et al.* (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*:9150-9155). Examples of insulator elements that can be included onto target gene expression vector in order to optimize expression include, but are not limited to:

1) chicken β-globin HS4 element (Prioleau *et al.* (1999) *EMBO J 18*: 4035-4048);

2) matrix attachment regions (MAR; see, *e.g.*, Ramakrishnan *et al.* (2000) *Mol Cell. Biol. 20*:868-877);

3) scaffold attachment regions (SAR; see, *e.g.*, Auten *et al.* (1999) *Human Gene Therapy 10*:1389-1399); and

4) universal chromatin opening elements (UCOE; WO/0005393 and WO/0224930)

The copy number of the target gene can be controlled by sequentially adding multiple target gene expression vectors containing the target gene onto multiple integration sites on the chromosome platform. Likewise, the copy number of the target gene can be controlled within an individual target gene expression vector by the addition of DNA sequences that promote gene amplification. For example, gene amplification can be induced utilizing the dihydrofolate reductase (DHFR) minigene with subsequent selection with methotrexate (see, *e.g.*, Schimke (1984) *Cell 37*:705-713) or amplification promoting sequences from the rDNA locus (see, *e.g.*, Wegner *et al.* (1989) *Nucl. Acids Res. 17*: 9909-9932).

6. Platforms with other recombinase system sites

A "double *lox*" targeting strategy mediated by Cre-recombinase (Bethke *et al.* (1997) *Nucl. Acids Res. 25*:2828-2834) can be used. This strategy employs a pair of heterospecific *lox* sites—*lox*A and *lox*B, which differ by one nucleotide in the 8 bp spacer region. Both sites are engineered into the artificial chromosome and also onto the targeting DNA vector. This allows for a direct site-specific insertion of a commercially relevant gene or genes by a Cre-catalyzed double crossover event. In essence a platform *ACes* is engineered with a hygromycin-resistance gene flanked by the double *lox* sites generating *lox-ACes*, which is maintained

in the thymidine kinase deficient cell, LMtk(-). The gene of interest, for example, for testing purposes, the green fluorescence protein gene, GFP and a HSV thymidine kinase gene (tk) marker, are engineered between the appropriate *lox* sites of the targeting vector. The vector DNA is cotransfected with plasmid pBS185 (Life Technologies) encoding the *Cre* recombinase gene into mammalian cells maintaining the dual-*lox* artificial chromosome. Transient expression of the *Cre* recombinase catalyzes the site-specific insertion of the gene and the tk-gene onto the artificial chromosome. The transfected cells are grown in HAT medium that selects for only those cells that have integrated and expressed the thymidine kinase gene. The HAT^R colonies are screened by PCR analyses to identify artificial chromosomes with the desired insertion.

To generate the *lox-ACes*, Lambda-Hyg^R-*lox* DNA is transfected into the LMtk(-) cell line harboring the precursor *ACes*. Hygromycin-resistant colonies are analyzed by FISH and Southern blotting for the presence of a single copy insert on the *ACes*.

To demonstrate the gene replacement technology, cell lines containing candidate *lox-ACes* are cotransfected with pTK-GFP-*lox* and pBS185 (encoding the *Cre* recombinase gene) DNA. After transfection, transient expression of plasmid pBS185 will provide sufficient burst of *Cre* recombinase activity to catalyze DNA recombination at the *lox* sites. Thus, a double crossover event between the *ACes* target and the exogenous targeting plasmid carrying the *lox*A and *lox*B permits the simple replacement of the hygromycin-resistance gene on the *lox-ACes* for the tk-GFP cassette from the targeting plasmid, with no integration of vector DNA. Transfected cells are grown in HAT-media to select for tk-expression. Correct targeting will result in the generation of HAT^R, hygromycin sensitive, and green fluorescent cells. The desired integration event is verified by Southern and PCR analyses. Specific PCR primer sets

are used to amplify DNA sequences flanking the individual *lox*A and *lox*B sites on the *lox-ACes* before and after homologous recombination.

D. Exemplary applications of the Platform *ACes*

Platform *ACes* are applicable and tractable for different/optimized cell lines. Those that include a fluorescent marker, for example, can be purified and isolated using fluorescent activated cell sorting (FACS), and subsequently delivered to a target cell. Those with selectable markers provide for efficient selection and provide a growth advantage. Platform *ACes* allow multiple payload delivery of donor target vectors via a positive-selection site-specific, recombination system, and they allow for the inclusion of additional genetic factors that improve protein production and protein quality.

The construction and use of the platform *ACes* as provided for each application may be similarly applied to other applications. Particular descriptions are for exemplification.

1. Cellular Protein Production Platform *ACes* (CPP *ACes*)

As described herein, *ACes* can be produced from acrocentric chromosomes in rodent (mouse, hamster) cell lines via megareplicator induced amplification of heterochromatin/rDNA sequences. Such *ACes* are ideal for cellular protein production as well as other applications described herein and known to those of skill in the art. *ACes* platforms that contain a plurality of recombination sites are particularly suitable for engineering as cellular protein production systems.

In one embodiment, CPP *ACes* involve a two-component system: the platform chromosome containing multiple engineering sites and the donor target vector containing a platform-specific recombination site with designed expression cassettes (see Figure 9).

The platform *ACes* can be produced from any artificial chromosome, particularly the amplification-based artificial chromosomes.

For exemplification, they are produced from rodent artificial chromosomes produced from acrocentric chromosomes using the technology of U.S. Patent Nos. 6,077,697 and 6,025,155 and published International PCT application No. WO 97/40183, in which nucleic acid is targeted to the pericentric heterochromatic, and, particularly into rDNA to initiate the replication event(s). The *ACes* can be produced directly in the chosen cellular protein production cell lines, such as, but not limited to, CHO cells, hybridomas, plant cells, plant tissues, plant protoplasts, stem cells and plant calli.

a. Platform Construction

In the exemplary embodiment, the initial *de novo* platform construction requires co-transfecting with excess targeting DNA, such as, rDNA or lambda DNA without an *attP* region, and an engineered selectable marker. The engineered selectable marker should contain promoter, generally a constitutive promoter, such as human, viral, i.e., adenovirus or SV40 promoter, including the human ferritin heavy chain promoter (SEQ ID NO:128), SV40 and EF1α promoters, to control expression of a marker gene that provides a selective growth advantage to the cell. An example of such a marker gene is the *E. coli hisD* gene (encoding histidinol dehydrogenase) which is homologous and analogous to the *S. typhimurium hisD* a dominant marker selection system for mammalian cells previously described (see, Hartman *et al.* (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*:8047-8051). Since histidine is an essential amino acid in mammals and a nutritional requirement in cell culture, the *E. coli hisD* gene can be used to select for histidine prototrophy in defined media. Furthermore more stringent selection can be placed on the cells by including histinol in the medium. Histidinol is itself permeable and toxic to cells. The *hisD* provides a means of detoxification.

Placed between the promoter and the marker gene is the bacteriophage lambda *attP* site to use the bacteriophage lambda integrase dependent site-specific recombination system (described herein). The insertion of an *attP* site downstream of a promoter element provide forward selection of site-specific recombination events onto the platform *ACes*.

b. Donor Target Vector Construction

A second component of the CPP platform *ACes* system involves the construction of donor target vectors containing a gene product(s) of interest for the CPP platform *ACes*. Individual donor target vectors can be designed for each gene product to be expressed thus enabling maximum usage of a *de novo* constructed platform *ACes*, so that one or a few CPP platform *ACes* will be required for many gene targets.

A key feature of the donor vector target is the *promoterless* marker gene containing an upstream *attB* site (marker 2 on figure 9). Normally the marker would not be expressed unless it is placed downstream of a promoter sequence. As discussed above, using the lambda integrase technology (λINT_{E174R} on Figure 8 and Figure 9), site-specific recombination between the *attB* site on the vector and the promoter-*attP* site on the CPP platform *ACes* result in the expression of the donor target vector marker providing positive selection for the site-specific event. Site-specific recombination events on the CPP *ACes* versus random integrations next to a promoter in the genome (false positive) can be quickly screened by designing primers to detect the correct event by PCR. In addition, since the lambda integrase reaction is unidirectional, i.e. excision reaction is not possible, a number of unique targets can be loaded onto the CPP platform *ACes* limited only by the number of markers available.

Additional features of the donor target vector include gene target expression cassettes flanked by either chromatin insulator regions, matrix attachment regions (MAR) or scaffold attachment regions (SAR). The use of these regions will provide a more "open" chromatin environment for gene expression and help alleviate silencing. An example of such a cassette for expressing a monoclonal antibody is described. For this purpose, a strong constitutive promoter, e.g. chicken β-actin or RNA PolI, is used to drive the expression of the heavy and light chain open reading frames. The heavy and light chain sequences flank a nonattenuated human IRES ($IRES_H$; from the 5'UTR of NRF1 gene; see Oumard et al., 2000, *Mol. and Cell Biol.*, 20(8):2755-2759) element thereby coordinating transcription of both heavy and light chain sequence. Distal to the light chain open reading frame resides an additional viral encoded IRES ($IRES_V$ modified ECMV internal ribosomal entry site (IRES)) element attenuating the expression of the fluorescent marker gene hrGFP from *Renilla* (Stratagene). By linking the hrGFP with an attenuated IRES, the heavy and light chains along with the hrGFP are monocistronic. Thus, the identification of hrGFP fluorescing cells will provide a means to detect protein producing cells. In addition, high producing cell lines can be identified and isolated by FACS thereby decreasing the time frame in finding high expressers. Functional monoclonal antibody will be confirmed by ELISA.

c. Additional components in cellular protein production platform *ACes* (CPP *Aces*)

In addition to the aforementioned CPP *ACes* system, other genetic factors can be included to enhance the yield and quality of the expressed protein. Again to provide maximum flexibility, these additional factors can be inserted onto the CPP platform *ACes* by λINTE174R dependent site-specific recombination. Other factors that could be used with a CPP

Platform *ACes* include for example, adenovirus E1a transactivation system which upregulates both cellular and viral promoters (see, e.g., Svensson and Akusjarvi (1984) EMBO 3:789-794; and US patents 5,866,359; 4,775,630 and 4,920,211).

d. Targets for CHO-*ACes* engineering to enhance cell growth, such as CHO cell growth and protein production/ quality

If adding these additional factors onto the CPP *ACes* is not prudent or desired, the host cell, CHO cells, can be engineered to express these factors (see, below, targets for CHO-*ACes* engineering to enhance CHO cell growth and protein production/quality). Additional factors to consider including are addition of insulin or IGF-1 to sustain viabililty; human sialyltransferases or related factors to produce more human-like glycoproteins; expression of factors to decrease ammonium accumulation during cell growth; expression of factors to inhibit apoptosis; expression of factors to improve protein secretion and protein folding; and expression of factors to permit serum-free transfection and selection.

1) Addition of insulin or IGF-1 to sustain viabililty

Stimulatory factors and/or their receptors are expressed to set up an autocrine loop, to improve cell growth, such as CHO cell growth. Two exemplary candidates are insulin and IGF-1 (see, Biotechnol Prog 2000 Sep;16(5):693-7). Insulin is the most commonly used growth factor for sustaining cell growth and viability in serum-free Chinese hamster ovary (CHO) cell cultures. Insulin and IGF-1 analog (LongR(3) serve as growth and viability factors for CHO cells.

CHO cells were modified to produce higher levels of essential nutrients and factors. A serum-free (SF) medium for dihydrofolate reductase-deficient Chinese hamster ovary cells (DG44 cells) was prepared. Chinese hamster ovary cells (DG44 cells), which are normally

maintained in 10% serum medium, were gradually weaned to 0.5% serum medium to increase the probability of successful growth in SF medium (see, Kim *et al.* (199) *In Vitro Cell Dev Biol Anim 35(4)*:178-82). A SF medium (SF-DG44) was formulated by supplementing the basal medium with these components; basal medium was prepared by supplementing Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 with hypoxanthine (10 mg/l) and thymidine (10 mg/l). Development of a SF medium for DG44 cells was facilitated using a Plackett-Burman design technique and weaning of cells.

2) Human sialyltransferases or related factors to produce more human-like glycoproteins

CHO cells have been modified by increasing their ability to process protein via addition of complex carbohydrates. This has been achieved by overexpression of relevant processing enzymes, or in some cases, reducing expression of relevant enzymes (see, Bragonzi *et al.* (2000) *Biochim Biophys Acta 1474(3)*:273-282; see, also Weikert *et al.* (1999) *Nature biotech. 17*:1116-11121; Ferrari J *et al.* (1998) *Biotechnol Bioeng 60(5)*:589-95). A CHO cell line expressing alpha2,6-sialyltransferase was developed for the production of human-like sialylated recombinant glycoproteins. The sialylation defect of CHO cells can be corrected by transfecting the alpha2,6-sialyltransferase (alpha2,6-ST) cDNA into the cells. Glycoproteins produced by such CHO cells display alpha2,6-and alpha2,3-linked terminal sialic acid residues, similar to human glycoproteins.

As another example for improving the production of human-like sialylated recombinant glycoproteins, a CHO cell line has been developed that constitutively expresses sialidase antisense RNA (see, Ferrari J *et al.* (1998) *Biotechnol Bioeng 60(5)*:589-95). Several antisense expression

vectors were prepared using different regions of the sialidase gene. Co-transfection of the antisense constructs with a vector conferring puromycin resistance gave rise to over 40 puromycin resistant clones that were screened for sialidase activity. A 5' 474 bp coding segment of the sialidase cDNA, in the inverted orientation in an SV 40-based expression vector, gave maximal reduction of the sialidase activity to about 40% wild-type values.

Oligosaccharide biosynthesis pathways in mammalian cells have been engineered for generation of recombinant glycoproteins (see, *e.g.*, Sburlati (1998) *Biotechnol Prog 14(2)*:189-92), which describes a Chinese hamster ovary (CHO) cell line capable of producing bisected oligosaccharides on glycoproteins. This cell line was created by overexpression of a recombinant N-acetylglucosaminyltransferase III (GnT-III) (see, also, Prati *et al.* (1998) *Biotechnol Bioeng 59(4)*:445-50, which describes antisense strategies for glycosylation engineering of CHO cells).

3) Expression of factors to decrease ammonium accumulation during cell growth

Excess ammonium, which is a by-product of CHO cell metabolism can have detrimental effects on cell growth and protein quality (see, Yang *et al.* (2000) *Biotechnol Bioeng 68(4)*:370-80). To solve this problem ammonium levels were modified by overexpressing carbamoyl phosphate synthetase I and ornithine transcarbamoylase or glutamine synthetase in CHO cells. Such modification resulted in reduced ammonium levels observed and an increase in the growth rate (see Kim *et al.* (2000) *J Biotechnol 81(2-3)*:129-40; and Enosawa *et al.* (1997) *Cell Transplant 6(5)*:537-40).

4) Expression of factors to improve protein secretion and protein folding

Overexpression of relevant enzymes can be engineered into the *ACes* to improve protein secretion and folding.

5) Expression of factors to permit serum-free transfection and selection

It is advantageous to have the ability to convert CHO cells in suspension growing in serum free medium to adherence with out having to resort to serum addition. Laminin or fibronectin addition is sufficient to make cells adherent (see, *e.g.*, Zaworski *et al.* (1993) *Biotechniques 15(5)*:863-6) so that expressing either of these genes in CHO cells under an inducible promoter should allow for reversible shift to adherence without requiring serum addition.

2. Platform *ACes* and Gene Therapy

The platform *ACes* provided herein are contemplated for use in mammalian gene therapy, particularly human gene therapy. Human *ACes* can be derived from human acrocentric chromosomes from human host cells, in which the amplified sequences are heterochromatic and/or human rDNA. Different platform *ACes* applicable for different tissue cell types are provided. The *ACes* for gene therapy can contain a single copy of a therapeutic gene inserted into a defined location on platform *ACes*. Therapeutic genes include genomic clones, cDNA, hybrid genes and other combinations of sequences. Preferred selectable markers are those from the mammalian host, such as human derived factors so that they are non-immunogenic, non-toxic and allow for efficient selection, such as by FACS and/or drug resistance.

Platform *ACes*, useful for gene therapy and other applications, as noted herein, can be generated by megareplicator dependent amplification, such as by the methods in U.S. Patent Nos. 6,077,697 and 6,025,155 and published International PCT application No. WO 97/40183. In one embodiment, human *ACes* are produced using

human rDNA constructs that target rDNA arrays on human acrocentric chromosomes and induce the megareplicator in human cells, particularly in primary cell lines (with sufficient number of doublings to form the *ACes*) or stem cells (such as hematopoietic stem cells, mesenchymal stem cells, adult stem cells or embryonic stem cells) to avoid the introduction of potentially harmful rearranged DNA sequences present in many transformed cell lines. Megareplicator induced *ACes* formation can result in multiple copies of targeting DNA/selectable markers in each amplification block on both chromosomal arms of the platform *ACes*.

In view of the considerations regarding immunogenicity and toxicity, the production of human platform *ACes* for gene therapy applications employs a two component system analogous to the platform *ACes* designed for cellular protein production (CPP platform *ACes*). The system includes a platform chromosome of entirely human DNA origin containing multiple engineering sites and a gene target vector carrying the therapeutic gene of interest.

a. Platform Construction

The initial *de novo* construction of the platform chromosome employs the co-transfection of excess targeting DNA and a selectable marker. In one embodiment, the DNA is targeted to the rDNA arrays on the human acrocentric chromosomes (chromosomes 13, 14, 15, 21 and 22). For example, two large human rDNA containing PAC clones 18714 and 18720 and the human PAC clone 558F8 are used for targeting (Genome Research (ML) now Incyte, BACPAC Resources, 747 52nd Street, Oakland CA). The mouse rDNA clone pFK161 (SEQ ID NO: 118), which was used to make the human SATAC from the 94-3 hamster/human hybrid cell line (see, *e.g.*, published International PCT application No. WO 97/40183 and Csonka, *et al, Journal of Cell Science*

113:3207-32161 and Example 1 for a description of pFK161) can also be used.

For animal applications, selectable markers should be non-immunogenic in the animal, such as a human, and include, but are not limited to: human nerve growth factor receptor (detected with a MAb, such as described in US patent 6,365,373); truncated human growth factor receptor (detected with MAb), mutant human dihydrofolate reductase (DHFR; fluorescent MTX substrate available); secreted alkaline phosphatase (SEAP; fluorescent substrate available); human thymidylate synthase (TS; confers resistance to anti-cancer agent fluorodeoxyuridine); human glutathione S-transferase alpha (GSTA1; conjugates glutathione to the stem cell selective alkylator busulfan; chemoprotective selectable marker in CD34+ cells); CD24 cell surface antigen in hematopoietic stem cells; human CAD gene to confer resistance to N-phosphonacetyl-L-aspartate (PALA); human multi-drug resistance-1 (MDR-1; P-glycoprotein surface protein selectable by increased drug resistance or enriched by FACS); human CD25 (IL-2α; detectable by Mab-FITC); Methylguanine-DNA methyltransferase (MGMT; selectable by carmustine); and Cytidine deaminase (CD; selectable by Ara-C).

Since megareplicator induced amplification generates multiple copies of the selectable marker, a second consideration for the selection of the human marker is the resulting dose of the expressed marker after *ACes* formation. High level of expression of certain markers may be detrimental to the cell and/or result in autoimmunity. One method to decrease the dose of the marker protein is by shortening its half-life, such as via the fusion of the well-conserved human ubiquitin tag (a 76 amino acid sequence) thus leading to increased turnover of the selectable marker. This has been used successfully for a number of reporter

systems including DHFR (see, *e.g.*, Stack *et al.* (2000) *Nature Biotechnology 18*:1298-1302 and references cited therein).

Using the ubiquitin tagged protein, a human selectable marker system analogous to the CPP *ACes* described herein is constructed. Briefly, a tagged selectable marker, such as for example one of those described herein, is cloned downstream of an *attP* site and expressed from a human promoter. Exemplary promoters contemplated for use herein include, but are not limited to, the human ferritin heavy chain promoter (SEQ ID NO:128); RNA PolI; EF1α; TR; glyceraldehyde-3-phosphate dehydrogenase core promoter (GAP); a GAP core promoter including a proximal insulin inducible element and the intervening GAP sequence; phosphofructokinase promoter; and phosphoglycerate kinase promoter. Also contemplated herein is an aldolase A promoter H1 & H2 (representing closely spaced transcriptional start sites) along with the proximal H enhancer. There are 4 promoters (e.g., transcriptional start sites) for this gene, each having different regulatory and tissue activity. The H (most proximal 2) promoters are ubiquitously expressed off the H enhancer. This resulting marker can then be co-transfected along with excess human rDNA targeting sequence into the host cells. An important criteria for the selection of the

recipient cells is sufficient number of cell doublings for the formation and detection of *ACes*. Accordingly, the co-transfections should be attempted in human primary cells that can be cultured for long periods of time, such as for example, stem cells (e.g., hematopoietic, mesenchymal, adult or embryonic stem cells), or the like. Additional cell types, include, but are not limited to: single gene transfected cells exhibiting increased life-span; over-expressing c-myc cells, e.g. MSU1.1 (Morgan et al., 1991, Exp. Cell Res., Nov;197(1):125-136); over-expressing telomerase lines,

such as TERT cells; SV40 large T-antigen transfected lines; tumor cell lines, such as HT1080; and hybrid human cell lines, such as the 94-3 hamster/human hybrid cell line.

b. Gene Target Vector

The second component of the GT platform *ACes* (GT *ACes*) system involves the use of engineered target vectors carrying the therapeutic gene of interest. These are introduced onto the GT platform *ACes* via site-specific recombination. As with the CPP *ACes*, the use of engineered target vectors maximizes the use of the *de novo* generated GT platform *ACes* for most gene targets. Furthermore, using lambda integrase technology, GT platform *ACes* containing multiple *attP* sites permits the opportunity to incorporate multiple therapeutic targets onto a single platform. This could be of value in cases where a defined therapy requires multiple gene targets, a single therapeutic target requires an additional gene regulatory factor or a GT *ACes* requires a "kill" switch.

Similar to the CPP *ACes*, a feature of the gene target vector is the *promoterless* marker gene containing an upstream *attB* site (marker 2 on Figure 9). Normally, the marker (in this case, a cell surface antigen that can be sorted by FACS would be ideal) would not be expressed unless it is placed downstream of a promoter sequence. Using the lambda integrase technology (λINT_{E174R} on figure 9), site-specific recombination between the *attB* site on the vector and the promoter- *attP* site on the GT platform *ACes* results in the expression of marker#2 on the gene target vector, i.e. positive selection for the site-specific event. Site-specific recombination events on the GT *ACes* versus random integrations next to a promoter in the genome (false positive) can be quickly screened by designing primers to detect the correct event by PCR.

For expression of the therapeutic gene, human specific promoters, such as a ferritin heavy chain promoter (SEQ ID NO:128); EF1*α* or RNA

PolI, are used. These promoters are for high level expression of a cDNA encoded therapeutic protein. In addition to expressing cDNA (or even hybrid cDNA/artificial intron constructs), the GT platform *ACes* are used for engineering and expressing large genomic fragments carrying therapeutic genes of interest expressed from native promoter sequences. This is of importance in situations where the therapy requires precise cell specific expression or in instances where expression is best achieved from genomic clones versus cDNA.

3. Selectable markers for use, for example, in Gene Therapy (GT)

The following are selectable markers that can be incorporated into human *ACes* and used for selection.

Dual Resistance to 4-Hydroperoxycyclophosphamide and Methotrexate by Retroviral Transfer of the Human Aldehyde Dehydrogenase Class 1 Gene and a Mutated Dihydrofolate Reductase Gene

The genetic transfer of drug resistance to hematopoietic cells is one approach to overcoming myelosuppression caused by high-dose chemotherapy. Because cyclophosphamide (CTX) and methotrexate (MTX) are commonly used non-cross-resistant drugs, generation of dual drug resistance in hematopoietic cells that allows dose intensification may increase anti-tumor effects and circumvent the emergence of drug-resistant tumors, a retroviral vector containing a human cytosolic ALDH-1-encoding DNA clone and a human doubly mutated DHFR-encoding clone (Phe22/Ser31; termed F/S in the description of constructs) to generate increased resistance to CTX and MTX were constructed (Takebe *et al.* (2001) *Mol Ther 3(1)*:88-96). This construct may be useful for protecting patients from high-dose CTX- and MTX-induced myelosuppression. *ACes* can be similarly constructed.

Multiple mechanisms of N-phosphonacetyl-L-aspartate resistance in human cell lines: carbamyl-P synthetase/aspartate transcarbamylase/dihydro-orotase gene amplification is frequent only when chromosome 2 is rearranged

Rodent cells resistant to N-phosphonacetyl-L-aspartate (PALA) invariably contain amplified carbamyl-P synthetase/aspartate transcarbamylase/dihydro-orotase (CAD) genes, usually in widely spaced tandem arrays present as extensions of the same chromosome arm that carries a single copy of CAD in normal cells (Smith *et al.* (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*:1816-21). In contrast, amplification of CAD is very infrequent in several human tumor cell lines. Cell lines with minimal chromosomal rearrangement and with unrearranged copies of chromosome 2 rarely develop intrachromosomal amplifications of CAD. These cells frequently become resistant to PALA through a mechanism that increases the aspartate transcarbamylase activity with no increase in CAD copy number, or they obtain one extra copy of CAD by forming an isochromosome 2p or by retaining an extra copy of chromosome 2. In cells with multiple chromosomal aberrations and rearranged copies of chromosome 2, amplification of CAD as tandem arrays from rearranged chromosomes is the most frequent mechanism of PALA resistance. All of these different mechanisms of PALA resistance are blocked in normal human fibroblasts. Thus, *ACes* with multiple copies of the CAD gene would provide PALA resistance.

Retroviral coexpression of thymidylate synthase and dihydrofolate reductase confers fluoropyrimidine and antifolate resistance

Retroviral gene transfer of dominant selectable markers into hematopoietic cells can be used to select genetically modified cells in vivo or to attenuate the toxic effects of chemotherapeutic agents. Fantz *et al.* ((1998) *Biochem Biophys Res Comm 243(1)*:6-12) have shown that

retroviral gene transfer of thymidylate synthase (TS) confers resistance to TS directed anticancer agents and that co-expression of TS and dihydrofolate reductase (DHFR) confers resistance to TS and DHFR cytotoxic agents. Retroviral vectors encoding *Escherichia coli* TS, human TS, and the Tyr-to-His at residue 33 variant of human TS (Y33HhTS) were constructed and fibroblasts transfected with these vectors conferred comparable resistance to the TS-directed agent fluorodeoxyuridine (FdUrd, approximately 4-fold). Retroviral vectors that encode dual expression of Y33HhTS and the human L22Y DHFR (L22YhDHFR) variants conferred resistance to FdUrd (3- to 5-fold) and trimetrexate (30- to 140-fold). A L22YhDHFR-Y33HhTS chimeric retroviral vector was also constructed and transduced cells were resistant to FdUrd (3-fold), AG337 (3-fold), trimetrexate (100-fold) and methotrexate (5-fold). These results show that recombinant retroviruses can be used to transfer the cDNA that encodes TS and DHFR and dual expression in transduced cells is sufficiently high to confer resistance to TS and DHFR directed anticancer agents. *ACes* can be similarly constructed.

Human CD34+ cells do not express glutathione S-transferases alpha

The expression of glutathione S-transferases alpha (GST alpha) in human hematopoietic CD34+ cells and bone marrow was studied using RT-PCR and immunoblotting (Czerwinski M, Kiem *et al.* (1997) *Gene Ther 4(3)*:268-70). The GSTA1 protein conjugates glutathione to the stem cell selective alkylator busulfan. This reaction is the major pathway of elimination of the compound from the human body. Human hematopoietic CD34+ cells and bone marrow do not express GSTA1 message, which was present at a high level in liver, an organ relatively resistant to busulfan toxicity in comparison to bone marrow. Similarly, baboon CD34+ cells and dog bone marrow do not express GSTA1. Thus, human

GSTA1 is a chemoprotective selectable marker in human stem cell gene therapy and could be employed in *ACes* construction.

Selection of retrovirally transduced hematopoietic cells using CD24 as a marker of gene transfer

Pawliuk *et al.* ((1994) *Blood 84(9)*:2868-2877) have investigated the use of a cell surface antigen as a dominant selectable marker to facilitate the detection and selection of retrovirally infected target cells. The small coding region of the human cell surface antigen CD24 (approximately 240 bp) was introduced into a myeloproliferative sarcoma virus (MPSV)-based retroviral vector, which was then used to infect day 4 5-fluorouracil (5-FU)-treated murine bone marrow cells. Within 48 hours of termination of the infection procedure CD24-expressing cells were selected by fluorescent-activated cell sorting (FACS) with an antibody directed against the CD24 antigen. Functional analysis of these cells showed that they included not only in vitro clonogenic progenitors and day 12 colony-forming unit-spleen but also cells capable of competitive long-term hematopoietic repopulation. Double-antibody labeling studies performed on recipients of retrovirally transduced marrow cells showed that some granulocytes, macrophages, erythrocytes, and, to a lesser extent, B and T lymphocytes still expressed the transduced CD24 gene at high levels 4 months later. No gross abnormalities in hematopoiesis were detected in mice repopulated with CD24-expressing cells. These results show that the use of the CD24 cell surface antigen as a retrovirally encoded marker permits rapid, efficient, and nontoxic selection *in vitro* of infected primary cells, facilitates tracking and phenotyping of their progeny, and provides a tool to identify elements that regulate the expression of transduced genes in the most primitive hematopoietic cells. *ACes* could be similarly constructed.

DeltahGHR, a biosafe cell surface-labeling molecule for analysis and selection of genetically transduced human cells

A selectable marker for retroviral transduction and selection of human and murine cells is known (see, Garcia-Ortiz *et al.* (2000) *Hum Gene Ther 11(2)*:333-46). The molecule expressed on the cell surface of the transduced population is a truncated version of human growth hormone receptor (deltahGHR), capable of ligand (hGH) binding, but devoid of the domains involved in signal triggering. The engineered molecule is stably expressed in the target cells as an inert protein unable to trigger proliferation or to rescue the cells from apoptosis after ligand binding. This new marker, has a wide application spectrum, since hGHR in the human adult is highly expressed only in liver cells, and lower levels have been reported in certain lymphocyte cell populations. The deltahGHR label has high biosafety potential, as it belongs to a well-characterized hormonal system that is nonessential in adults, and there is extensive clinical experience with hGH administration in humans. The differential binding properties of several monoclonal antibodies (MAbs) are used in a cell rescue method in which the antibody used to select deltahGHR-transduced cells is eluted by competition with hGH or, alternatively biotinylated hGH is used to capture tagged cells. In the latter system, the final purified population is recovered free of attached antibodies in hGH (a substance approved for human use)-containing medium. Such a system could be used to identify *ACes* containing cells.

4. Transgenic models for evaluation of genes and discovery of new traits in plants

Of interest is the use of plants and plant cells containing artificial chromosomes for the evaluation of new genetic combinations and discovery of new traits. Artificial chromosomes, by virtue of the fact that they can contain significant amounts of DNA can also therefore encode

numerous genes and accordingly a multiplicity of traits. It is contemplated here that artificial chromosomes, when formed from one plant species, can be evaluated in a second plant species. The resultant phenotypic changes observed, for example, can indicate the nature of the genes contained within the DNA contained within the artificial chromosome, and hence permit the identification of novel genetic activities. Artificial chromosomes containing euchromatic DNA or partially containing euchromatic DNA can serve as a valuable source of new traits when transferred to an alien plant cell environment. For example, it is contemplated that artificial chromosomes derived from dicot plant species can be introduced into monocot plant species by transferring a dicot artificial chromosome. The dicot artificial chromosome possessing a region of euchromatic DNA containing expressed genes.

The artificial chromosomes can be designed to allow the artificial chromosome to recombine with the naturally occurring plant DNA in such a fashion that a large region of naturally occurring plant DNA becomes incorporated into the artificial chromosome. This allows the artificial chromosome to contain new genetic activities and hence carry novel traits. For example, an artificial chromosome can be introduced into a wild relative of a crop plant under conditions whereby a portion of the DNA present in the chromosomes of the wild relative is transferred to the artificial chromosome. After isolation of the artificial chromosome, this naturally occurring region of DNA from the wild relative, now located on the artificial chromosome can be introduced into the domesticated crop species and the genes encoded within the transferred DNA expressed and evaluated for utility. New traits and gene systems can be discovered in this fashion. The artificial chromosome can be modified to contain sequences that promote homologous recombination within plant cells, or

be modified to contain a genetic system that functions as a site-specific recombination system.

Artificial chromosomes modified to recombine with plant DNA offer many advantages for the discovery and evaluation of traits in different plant species. When the artificial chromosome containing DNA from one plant species is introduced into a new plant species, new traits and genes can be introduced. This use of an artificial chromosome allows for the ability to overcome the sexual barrier that prevents transfer of genes from one plant species to another species. Using artificial chromosomes in this fashion allows for many potentially valuable traits to be identified including traits that are typically found in wild species. Other valuable applications for artificial chromosomes include the ability to transfer large regions of DNA from one plant species to another, such as DNA encoding potentially valuable traits such as altered oil, carbohydrate or protein composition, multiple genes encoding enzymes capable of producing valuable plant secondary metabolites, genetic systems encoding valuable agronomic traits such as disease and insect resistance, genes encoding functions that allow association with soil bacterium such as growth promoting bacteria or nitrogen fixing bacteria, or genes encoding traits that confer freezing, drought or other stress tolerances. In this fashion, artificial chromosomes can be used to discover regions of plant DNA that encode valuable traits.

The artificial chromosome can also be designed to allow the transfer and subsequent incorporation of these valuable traits now located on the artificial chromosome into the natural chromosomes of a plant species. In this fashion the artificial chromosomes can be used to transfer large regions of DNA encoding traits normally found in one plant species into another plant species. In this fashion, it is possible to derive a plant cell that no longer needs to carry an artificial chromosome to

posses the novel trait. Thus, the artificial chromosome would serve as the transfer mechanism to permit the formation of plants with greater degree of genetic diversity.

The design of an artificial chromosome to accomplish the afore-mentioned purposes can include within the artificial chromosome the presence of specific DNA sequences capable of acting as sites for homologous recombination to take place. For example, the DNA sequence of *Arabidopsis* is now known. To construct an artificial chromosome capable of recombining with a specific region of *Arabidopsis* DNA, a sequence of *Arabidopsis* DNA, normally located near a chromosomal location encoding genes of potential interest can be introduced into an artificial chromosome by methods provided herein. It may be desirable to include a second region of DNA within the artificial chromosome that provides a second flanking sequence to the region encoding genes of potential interest, to promote a double recombination event which would ensure transfer of the entire chromosomal region, encoding genes of potential interest, to the artificial chromosome. The modified artificial chromosome, containing the DNA sequences capable of homologous recombination region, can then be introduced into *Arabidopsis* cells and the homologous recombination event selected.

It is convenient to include a marker gene to allow for the selection of a homologous recombination event. The marker gene is preferably inactive unless activated by an appropriate homologous recombination event. For example, US 5,272,071, describes a method where an inactive plant gene is activated by a recombination event such that desired homologous recombination events can be easily scored. Similarly, US 5,501,967 describes a method for the selection of homologous recombination events by activation of a silent selection gene first introduced into the plant DNA, the gene being activated by an appropriate

homologous recombination event. Both of these methods can be applied to enable a selective process to be included to select for recombination between an artificial chromosome and a plant chromosome. Once the homologous recombination event is detected, the artificial chromosome, once selected, is isolated and introduced into a recipient cell, for example, tobacco, corn, wheat or rice, and the expression of the newly introduced DNA sequences evaluated.

Phenotypic changes in the recipient plant cells containing the artificial chromosome, or in regenerated plants containing the artificial chromosome, allows for the evaluation of the nature of the traits encoded by the *Arabidopsis* DNA, under conditions naturally found in plant cells, including the naturally occurring arrangement of DNA sequences responsible for the developmental control of the traits in the normal chromosomal environment.

Traits such as durable fungal or bacterial disease resistance, new oil and carbohydrate compositions, valuable secondary metabolites such as phytosterols, flavonoids, efficient nitrogen fixation or mineral utilization, resistance to extremes of drought, heat or cold are all found within different populations of plant species and are often governed by multiple genes. The use of single gene transformation technologies does not permit the evaluation of the multiplicity of genes controlling many valuable traits. Thus, incorporation of these genes into artificial chromosomes allows the rapid evaluation of the utility of these genetic combinations in heterologous plant species.

The large scale order and structure of the artificial chromosome provides a number of unique advantages in screening for new utilities or novel phenotypes within heterologous plant species. The size of new DNA that can be carried by an artificial chromosome can be millions of base pairs of DNA, representing potentially numerous genes that may

have novel utility in a heterologous plant cell. The artificial chromosome is a "natural" environment for gene expression, the problems of variable gene expression and silencing seen for genes transferred by random insertion into a genome should not be observed. Similarly, there is no need to engineer the genes for expression, and the genes inserted would not need to be recombinant genes. Thus, one expects the expression from the transferred genes to be temporal and spatial, as observed in the species from where the genes were initially isolated. A valuable feature for these utilities is the ability to isolate the artificial chromosomes and to further isolate, manipulate and introduce into other cells artificial chromosomes carrying unique genetic compositions.

Thus, the use of artificial chromosomes and homologous recombination in plant cells can be used to isolate and identify many valuable crop traits.

In addition to the use of artificial chromosomes for the isolation and testing of large regions of naturally occurring DNA, methods for the use of artificial chromosomes and cloned DNA are also contemplated. Similar to that described above, artificial chromosomes can be used to carry large regions of cloned DNA, including that derived from other plant species.

The ability to incorporate novel DNA elements into an artificial chromosome as it is being formed allows for the development of artificial chromosomes specifically engineered as a platform for testing of new genetic combinations, or "genomic" discoveries for model species such as *Arabidopsis*. It is known that specific "recombinase" systems can be used in plant cells to excise or re-arrange genes. These same systems can be used to derive new gene combinations contained on an artificial chromosome.

The artificial chromosomes can be engineered as platforms to accept large regions of cloned DNA, such as that contained in Bacterial

Artificial Chromosomes (BACs) or Yeast Artificial Chromosomes (YACs). It is further contemplated, that as a result of the typical structure of artificial chromosomes containing tandemly repeated DNA blocks, that sequences other than cloned DNA sequence can be introduced by recombination processes. In particular recombination within a predefined region of the tandemly repeated DNA within the artificial chromosome provides a mechanism to "stack" numerous regions of cloned DNA, including large regions of DNA contained within BACs or YACs clones. Thus, multiple combinations of genes can be introduced onto artificial chromosomes and these combinations tested for functionality. In particular, it is contemplated that multiple YACs or BACs can be stacked onto an artificial chromosomes, the BACs or YACs containing multiple genes of complex pathways or multiple genetic pathways. The BACs or YACs are typically selected based on genetic information available within the public domain, for example from the Arabidopsis Information Management System (http://aims.cps.msu.edu/aims/index.html) or the information related to the plant DNA sequences available from the Institute for Genomic Research (http://www.tigr.org) and other sites known to those skilled in the art. Alternatively, clones can be chosen at random and evaluated for functionality. It is contemplated that combinations providing a desired phenotype can be identified by isolation of the artificial chromosome containing the combination and analyzing the nature of the inserted cloned DNA.

In this regard, it is contemplated that the use of site-specific recombination sequences can have considerable utility in developing artificial chromosomes containing DNA sequences recognized by recombinase enzymes and capable of accepting DNA sequences containing same. The use of site-specific recombination as a means to target an introduced DNA to a specific locus has been demonstrated in

the art and such methods can be employed. The recombinase systems can also be used to transfer the cloned DNA regions contained within the artificial chromosome to the naturally occurring plant or mammalian chromosomes.

As noted herein, many site-specific recombinases are known and can be identified (Kilby *et al.* (1993) *Trends in Genetics 9*:413-418). The three recombinase systems that have been extensively employed include: an activity identified as R encoded by the pSR1 plasmid of *Zygosaccharomyes rouxii*, FLP encoded for the 2um circular plasmid from *Saccharomyces cerevisiae* and *Cre-lox* from the phage P1.

The integration function of site-specific recombinases is contemplated as a means to assist in the derivation of genetic combinations on artificial chromosomes. In order to accomplish this, it is contemplated that a first step of introducing site-specific recombinase sites into the genome of a plant cell in an essentially random manner is conducted, such that the plant cell has one or more site-specific recombinase recognition sequences on one or more of the plant chromosomes. An artificial chromosome is then introduced into the plant cell, the artificial chromosome engineered to contain a recombinase recognition site (e.g., integration site) capable of being recognized by a site-specific recombinase. Optionally, a gene encoding a recombinase enzyme is also included, preferably under the control of an inducible promoter. Expression of the site-specific recombinase enzyme in the plant cell, either by induction of a inducible recombinase gene, or transient expression of a recombinase sequence, causes a site-specific recombination event to take place, leading to the insertion of a region of the plant chromosomal DNA (containing the recombinase recognition site) into the recombinase recognition site of the artificial chromosome, and forming an artificial chromosome containing plant chromosomal DNA.

The artificial chromosome can be isolated and introduced into a heterologous host, preferably a plant host, and expression of the newly introduced plant chromosomal DNA can be monitored and evaluated for desirable phenotypic changes. Accordingly, carrying out this recombination with a population of plant cells wherein the chromosomally located recombinase recognition site is randomly scattered throughout the chromosomes of the plant, can lead to the formation of a population of artificial chromosomes, each with a different region of plant chromosomal DNA, and each potentially representing a novel genetic combination.

This method requires the precise site-specific insertion of chromosomal DNA into the artificial chromosome. This precision has been demonstrated in the art. For example, Fukushige and Sauer ((1992) Proc. Natl. Acad. Sci. USA, 89:7905-7909) demonstrated that the *Cre-lox* homologous recombination system could be successfully employed to introduce DNA into a predefined locus in a chromosome of mammalian cells. In this demonstration a promoter-less antibiotic resistance gene modified to include a *lox* sequence at the 5′ end of the coding region was introduced into CHO cells. Cells were re-transformed by electroporation with a plasmid that contained a promoter with a *lox* sequence and a transiently expressed *Cre* recombinase gene. Under the conditions employed, the expression of the *Cre* enzyme catalyzed the homologous recombination between the *lox* site in the chromosomally located promoter-less antibiotic resistance gene, and the *lox* site in the introduced promoter sequence, leading to the formation of a functional antibiotic resistance gene. The authors demonstrated efficient and correct targeting of the introduced sequence, 54 of 56 lines analyzed corresponded to the predicted single copy insertion of the DNA due to *Cre* catalyzed site-specific homologous recombination between the *lox* sequences.

Accordingly a *lox* sequence may be first added to a genome of a plant species capable of being transformed and regenerated to a whole plant to serve as a recombinase target DNA sequence for recombination with an artificial chromosome. The *lox* sequence may be optimally modified to further contain a selectable marker which is inactive but can be activated by insertion of the *lox* recombinase recognition sequence into the artificial chromosome.

A promoterless marker gene or selectable marker gene linked to the recombinase recognition sequence, which is first inserted into the chromosomes of a plant cell can be used to engineer a platform chromosome. A promoter is linked to a recombinase recognition site, in an orientation that allows the promoter to control the expression of the marker or selectable marker gene upon recombination within the artificial chromosome. Upon a site-specific recombination event between a recombinase recognition site in a plant chromosome and the recombinase recognition site within the introduced artificial chromosome, a cell is derived with a recombined artificial chromosome, the artificial chromosome containing an active marker or selectable marker activity that permits the identification and or selection of the cell.

The artificial chromosomes can be transferred to other plant or animal species and the functionality of the new combinations tested. The ability to conduct such an inter-chromosomal transfer of sequences has been demonstrated in the art. For example, the use of the *Cre-lox* recombinase system to cause a chromosome recombination event between two chromatids of different chromosomes has been shown.

Any number of recombination systems may be employed as described herein, such as, but not limited to, bacterially derived systems such as the att/int system of phage lambda, and the Gin/gix system.

More than one recombination system may be employed, including, for example, one recombinase system for the introduction of DNA into an artificial chromosome, and a second recombinase system for the subsequent transfer of the newly introduced DNA contained within an artificial chromosome into the naturally occurring chromosome of a second plant species. The choice of the specific recombination system used will be dependent on the nature of the modification contemplated.

By having the ability to isolate an artificial chromosome, in particular, artificial chromosomes containing plant chromosomal DNA introduced via site-specific recombination, and re-introduce the chromosome into other mammalian or plant cells, particularly plant cells, these new combinations can be evaluated in different crop species without the need to first isolate and modify the genes, or carry out multiple transformations or gene transfers to achieve the same combination isolation and testing combinations of the genes in plants. The use of a site-specific recombinase also allows the convenient recovery of the plant chromosomal region into other recombinant DNA vectors and systems, such as mammalian or insect systems, for manipulation and study.

Also contemplated herein are *ACes*, cell lines and methods for use in screening a new chromosomal combinations, deletions, truncations with eucaryotic genome that take advantage of the site-specific recombination systems incorporated onto platform *ACes* provided herein. For example, provided herein is a cell line useful for making a library of *ACes*, comprising a multiplicity of heterologous recombination sites randomly integrated throughout the endogenous chromosomes. Also provided herein is a method of making a library of *ACes* comprising random portions of a genome, comprising introducing one or more *ACes* into a cell line comprising a multiplicity of heterologous recombination

sites randomly integrated throughout the endogenous chromosomes, under conditions that promote the site-specific chromosomal arm exchange of the *ACes* into, and out of, a multiplicity of the heterologous recombination sites within the cell's chromosomal DNA; and isolating said multiplicity of *ACes*, thereby producing a library of *ACes* whereby multiple *ACes* have different portions of the genome within. Also provided herein is a library of cells useful for genomic screening, said library comprising a multiplicity of cells, wherein each cell comprises an *ACes* having a mutually exclusive portion of a chromosomal nucleic acid therein. The library of cells can be from a different species and/or cell type than the chromosomal nucleic acid within the *ACes*. Also provided is a method of making one or more cell lines, comprising

a) integrating into endogenous chromosomal DNA of a selected cell species, a multiplicity of heterologous recombination sites,

b) introducing a multiplicity of *ACes* under conditions that promote the site-specific chromosomal arm exchange of the *ACes* into, and out of, a multiplicity of the heterologous recombination sites integrated within the cell's endogenous chromosomal DNA;

c) isolating said multiplicity of *ACes*, thereby producing a library of *ACes* whereby a multiplicity of *ACes* have mutually exclusive portions of the endogenous chromosomal DNA therein;

d) introducing the isolated multiplicity of *ACes* of step c) into a multiplicity of cells, thereby creating a library of cells;

e) selecting different cells having mutually exclusive *ACes* therein and clonally expanding or differentiating said different cells into clonal cell cultures, thereby creating one or more cell lines.

These *ACes*, cell lines and methods utilize the site-specific recombination sites on platform *ACes* analogous YAC manipulation related to: the methods of generating terminal deletions in normal and

artificial chromosomes (e.g., *ACes*; as described in Vollrath et al., 1988, *PNAS, USA*, 85:6027-66031; and Pavan et al., *PNAS, USA*, 87:1300-1304); the methods of generating interstitial deletions in normal and artificial chromosomes (as described in Campbell et al., 1991, *PNAS, USA*, 888:5744-5748); and the methods of detecting homologous recombination between two *ACes* (as described in Cellini et al., 1991, *Nuc. Acid Res.*, 19(5):997-1000).

5. Use of plateform *ACes* in Pharmacogenomic/toxicology applications (development of "Reporter *ACes*")

In addition to the placement of genes onto *ACes* chromosomes for therapeutic protein production or gene therapy, the platform can be engineered via the IntR lambda integrase to carry reporter-linked constructs (reporter genes) that monitor changes in cellular physiology as measured by the particular reporter gene (or a series of different reporter genes) readout. The reporter linked constructs are designed to include a gene that can be detected (by for example fluorescence, drug resistance, immunohistochemistry, or transcript production, and the like) with well-known regulatory sequences that would control the expression of the detectable gene. Exemplary regulatory promoter sequences are well-known in the art.

A) Reporter *ACes* for drug pathway screening

The *ACes* can be engineered to carry reporter-linked constructs that indicate a signal is being transduced through one or a number of pathways. For example, transcriptionally regulated promoters from genes at the end (or any other chosen point) of particular signal transduction pathways could be engineered on the *ACes* to express the appropriate readout (either by fluorescent protein production or drug resistance) when the pathway is activated (or down-regulated as well). In one embodiment, a number of reporters from different pathways can be placed on an

ACes chromosome. Cells (and/or whole animals) containing such a Reporter *ACes* could be exposed to a variety of drugs or compounds and monitored for the effects of the drugs or compounds upon the selected pathway(s) by the reporter gene(s). Thus, drugs or compounds can be classified or identified by particular pathways they excite or down-regulate. Similarly, transcriptional profiles obtained from genomic array experiments can be biologically validated using the reporter *ACes* provided herein.

B) Reporter *ACes* for toxic compound testing

Environmental or man-made genotoxicants can be tested in cell lines carrying a number of reporter-genes platform *ACes* linked to promoters that are transcriptionally regulated in response to DNA damage, induced apoptosis or necrosis, and cell-cycle perturbations. Furthermore, new drugs and/or compounds could be tested in a similar manner with the genotoxicant *ACes* reporter for their cellular/genetic toxicity by such a screen. Likewise, toxic compound testing could be carried out in whole transgenic animals carrying the *ACes* chromosome that measures genotoxicant exposure ("canary in a coal mine"). Thus, the same or similar type *ACes* could be used for toxicity testing in either a cell-based or whole animal setting. An example would include *ACes* that carry reporter-linked genes controlled by various cytochrome P450 profiled promoters and the like.

C) Reporter *ACes* for individualized pharmacogenomics/drug profiling

A common disease may arise via various mechanisms. In many instances there are multiple treatments available for a given disease. However, the success of a given treatment may depend upon the mechanism by which the disease originated and/or by the genetic background of the patient. In order to establish the most effective

treatment for a given patient one could utilize the *ACes* reporters provided herein. *ACes* reporters can be used in patient cell samples to determine an individualized drug regimen for the patient. In addition, potential polymorphisms affecting the transcriptional regulation of an individual's particular gene can be assessed by this approach.

D) Reporter *ACes* for classification of similar patient tumors

As with other diseases as described in 5.C) above, cancer cells arise via different mechanisms. Furthermore, as a cancerous cell propagates it may undergo genomic alterations. An *ACes* reporter transferred to cells of different patients having the same disease, i.e. similar cancers, could be used to categorize the particular cancer of each patient, thereby facilitating the identification of the most effective therapeutic regimen. Examples would include the validation of array profiling of certain classes of breast cancers. Subsequently, appropriate drug profiling could be carried out as described above.

E) Reporter *ACes* as a "differentiation" sensor

Using the *ACes* reporter as a "differentiation" sensor in stem cells or other progenitor cells in order to enrich by selection (either FACS based screening, drug selection and/or use of suicide gene) for a particular class of differentiated or undifferentiated cells. For example, in one embodiment, this assay could also be used for compound screening for small molecule modifiers of cell differentiation.

F) Whole animal studies with Reporter *ACes*

Finally, with whole-body fluorescence imaging technology (Yang et al. (2000) PNAS 97:12278) any of the above Reporter *ACes* methods could be used in conjunction with whole-body imaging to monitor reporter genes within whole animals without sacrificing the animal. This would allow temporal and spatial analysis of expression patterns under a given set of conditions. The conditions tested may include for example, normal

differentiation of a stem cell, response to drug or compound treatment whether targeted to the diseased tissue or presented systemically, response to genotoxicants, and the like.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1

pFK161

Cosmid pFK161 (SEQ ID NO: 118) was obtained from Dr. Gyula Hadlaczky and contains a 9 kb *NotI* insert derived from a murine rDNA repeat (see clone 161 described in PCT Application Publication No. WO97/40183 by Hadlaczky *et al.* for a description of this cosmid). This cosmid, referred to as clone 161 contains sequence corresponding to nucleotides 10,232-15,000 in SEQ ID NO. 26. It was produced by inserting fragments of the megachromosome (see, U.S. Patent No. 6,077,697 and International PCT application No. WO 97/40183). For example, H1D3, which was deposited at the European Collection of Animal Cell Culture (ECACC) under Accession No. 96040929, is a mouse-hamster hybrid cell line carrying this megachromosome into plasmid pWE15 (Stratagene, La Jolla, California; SEQ ID No. 31) as follows. Half of a 100 μl low melting point agarose block (mega-plug) containing isolated SATACs was digested with *NotI* overnight at 37°C. Plasmid pWE15 was similarly digested with *NotI* overnight. The mega-plug was then melted and mixed with the digested plasmid, ligation buffer and T4 DNA ligase. Ligation was conducted at 16°C overnight. Bacterial DH5α cells were transformed with the ligation product and transformed cells were plated onto LB/Amp plates. Fifteen to twenty colonies were grown on each plate for a total of 189 colonies. Plasmid DNA was isolated from colonies that survived growth on LB/Amp medium and analyzed by Southern blot hybridization for the presence of DNA that

hybridized to a pUC19 probe. This screening methodology assured that all clones, even clones lacking an insert but yet containing the pWE15 plasmid, would be detected.

Liquid cultures of all 189 transformants were used to generate cosmid minipreps for analysis of restriction sites within the insert DNA. Six of the original 189 cosmid clones contained an insert. These clones were designated as follows: 28 (~9-kb insert), 30 (~ 9-kb insert), 60 (~4-kb insert), 113 (~9-kb insert), 157 (~9-kb insert) and 161 (~9-kb insert). Restriction enzyme analysis indicated that three of the clones (113, 157 and 161) contained the same insert.

For sequence analysis the insert of cosmid clone no. 161 was subcloned as follows. To obtain the end fragments of the insert of clone no. 161, the clone was digested with *NotI* and *BamHI* and ligated with *NotI/BamHI*-digested pBluescript KS (Stratagene, La Jolla, California). Two fragments of the insert of clone no. 161 were obtained: a 0.2-kb and a 0.7-kb insert fragment. To subclone the internal fragment of the insert of clone no. 161, the same digest was ligated with *BamHI*-digested pUC19. Three fragments of the insert of clone no. 161 were obtained: a 0.6-kb, a 1.8-kb and a 4.8-kb insert fragment.

The insert corresponds to an internal section of the mouse ribosomal RNA gene (rDNA) repeat unit between positions 7551-15670 as set forth in GENBANK accession no. X82564, which is provided as SEQ ID NO. 18. The sequence data obtained for the insert of clone no. 161 is set forth in SEQ ID NOS. 19-25. Specifically, the individual subclones corresponded to the following positions in GENBANK accession no. X82564 (SEQ ID NO:18) and in SEQ ID NOs. 19-25:

Subclone	Start	End	Site	SEQ ID No.
	in X82564			
161k1	7579	7755	*Not*I, *Bam*HI	19
161m5	7756	8494	*Bam*HI	20
161m7	8495	10231	*Bam*HI	21(shows only sequence corresponding to nt. 8495-8950), 22 (shows only sequence corresponding to nt. 9851- 10231)
161m12	10232	15000	*Bam*HI	23 (shows only sequence corresponding to nt. 10232-10600), 24 (shows only sequence corresponding to nt. 14267-15000)
161k2	15001	15676	*Not*I, *Bam*HI	25

The sequence set forth in SEQ ID NOs. 19-25 diverges in some positions from the sequence presented in positions 7551-15670 of GENBANK accession no. X82564. Such divergence may be attributable to random mutations between repeat units of rDNA.

For use herein, the rDNA insert from the clone was prepared by digesting the cosmid with *Not*I and *Bgl*II and was purified as described above. Growth and maintenance of bacterial stocks and purification of plasmids were performed using standard well known methods (see, *e.g.*, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory Press), and plasmids were purified from bacterial cultures using Midi - and Maxi-preps Kits (Qiagen, Mississauga, Ontario).

pDsRed1N1

This vector is available from Clontech (see SEQ ID No. 29) and encodes the red fluorescent protein (DsRed; Genbank accession no. AF272711; SEQ ID Nos. 39 and 40). DsRed, which has a vivid red fluorescence, was isolated from the IndoPacific sea anemone relative *Discosoma* species. The plasmid pDsRed1N1 (Clontech; SEQ ID No. 29) constitutively expresses a human codon-optimized variant of the

fluorescent protein under control of the CMV promoter. Unmodified, this vector expresses high levels of DsRed1 and includes sites for creating N-terminal fusions by cloning proteins of interest into the multiple cloning site (MCS). It is Kan and Neo resistant for selection in bacterial or eukaryotic cells.

Plasmid pMG

Plasmid pMG (InvivoGen, San Diego, California; see SEQ. ID. NO. 27 for the nucleotide sequence of pMG) contains the hygromycin phosphotransferase gene under the control of the immediate-early human cytomegalovirus (hCMV) enhancer/promoter with intron A. Vector pMG also contains two transcriptional units allowing for the coexpression of two heterologous genes from a single vector sequence.

The first transcriptional unit of pMG contains a multiple cloning site for insertion of a gene of interest, the hygromycin phosphotransferase gene (*hph*) and the immediate-early human cytomegalovirus (hCMV) enhancer/promoter with intron A (see, *e.g.*, Chapman *et al.* (1991) *Nuc. Acids Res. 19*:3979-3986) located upstream of *hph* and the multiple cloning site, which drives the expression of *hph* and any gene of interest inserted into the multiple cloning site as a polycistronic mRNA. The first transcriptional unit also contains a modified EMCV internal ribosomal entry site (IRES) upstream of the *hph* gene but downstream of the hCMV promoter and MCS for ribosomal entry in translation of the *hph* gene (see SEQ ID NO. 27, nucleotides 2736-3308). The IRES is modified by insertion of the constitutive *E. coli* promoter (EM7) within an intron (IM7) into the end of the IRES. In mammalian cells, the *E. coli* promoter is treated as an intron and is spliced out of the transcript. A polyadenylation signal from the bovine growth hormone (bGh) gene (see, *e.g.*, Goodwin and Rottman (1992) *J. Biol. Chem. 267*:16330-16334) and a pause site derived from the 3′ flanking region of the human $\alpha 2$

globin gene (see, *e.g.*, Enriquez-Harris *et al.* (1991) *EMBO J. 10*:1833-1842) are located at the end of the first transcription unit. Efficient polyadenylation is facilitated by inserting the flanking sequence of the bGh gene 3' to the standard AAUAAA hexanucleotide sequence.

The second transcriptional unit of pMG contains another multiple cloning site for insertion of a gene of interest and an EF-1α/HTLV hybrid promoter located upstream of this multiple cloning site, which drives the expression of any gene of interest inserted into the multiple cloning site. The hybrid promoter is a modified human elongation factor-1 alpha (EF-1 alpha) gene promoter (see, *e.g.*, Kim *et al.* (1990) *Gene 91*:217-223) that includes the R segment and part of the U5 sequence (R-U5') of the human T-cell leukemia virus (HTLV) type I long terminal repeat (see, *e.g.*, Takebe *et al.* (1988) *Mol. Cell. Biol 8*:466-472). The Simian Virus 40 (SV40) late polyadenylation signal (see Carswell and Alwine (1989) *Mol. Cell. Biol. 9*:4248-4258) is located downstream of the multiple cloning site. Vector pMG contains a synthetic polyadenylation site for the first and second transcriptional units at the end of the transcriptional unit based on the rabbit β-globin gene and containing the AATAAA hexanucleotide sequence and a GT/T-rich sequence with 22-23 nucleotides between them (see, *e.g.*, Levitt *et al.* (1989)*Genes Dev. 3*:1019-1025). A pause site derived from the C2 complement gene (see, Moreira *et al.* (1995) *EMBO J. 14*:3809-3819) is also located at the 3' end of the second transcriptional unit.

Vector pMG also contains an ori sequence (ori pMB1) located between the SV40 polyadenylation signal and the synthetic polyadenylation site.

EXAMPLE 2

A. Construction of targeting vector and transfection into LMtk- cells for the generation of platform chromosomes

A targeting vector derived from the vector pWE15 (GeneBank Accession # X65279) was modified by replacing the *Sal*I (Klenow filled)/*Sma*I neomycin resistance containing fragment with the *Pvu*II/*Bam*HI (Klenow filled) puromycin resistance containing fragment (isolated from plasmid pPUR, Clontech Laboratories, Inc. Palo Alto, CA; SEQ ID No. 30) resulting in plasmid pWEPuro. Subsequently a 9 Kb *Not*I fragment from the plasmid pFK161 (SEQ ID NO: 118) containing a portion of the mouse rDNA region was cloned into the *Not*I site of pWEPuro resulting in plasmid pWEPuro9K (Figure 2). The vector pWEPuro9K was digested with *Spe*I to linearize and transfected into LMtk- mouse cells. Puromycin resistant colonies were isolated and subsequently tested for artificial chromosome formation via fluorescent *in situ* hybridization (FISH) (using mouse major and minor DNA repeat sequences, the puromycin gene and telomeres sequences as probes), and fluorescent activated cell sorting (FACS). From this sort, a subclone was isolated containing an artificial chromosome, designated 5B11.12, which carries 4-8 copies of the puromycin resistance gene contained on the pWEPuro9K vector. FISH analysis of the 5B11.12 subclone demonstrated the presence of telomeres and mouse minor on the *ACes*. DOT PCR has been done on the 5B11.12 *ACes* revealing the absence of uncharacterized euchromatic regions on the *ACes*. A recombination site, such as an *att* or *loxP* engineering site or a plurality thereof, was introduced onto this *ACes* thereby providing a platform for site-specific introduction of heterologous nucleic acid.

B. Targeting a single sequence specific recombination site onto platform chromosomes

After the generation of the 5B11.12 platform, a single sequence-specific recombination site is placed onto the platform chromosome via homologous recombination. For this, DNA sequences containing the site-

specific recombination sequence can be flanked with DNA sequences of homology to the platform chromosome. For example, using the platform chromosome made from the pWEPuro9K vector, mouse rDNA sequences or mouse major satellite DNA can be used as homologous sequences to target onto the platform chromosome. A vector is designed to have these homologous sequences flanking the site-specific recombination site and, after the appropriate restriction enzyme digest to generate free ends of homology to the platform chromosome, the DNA is transfected into cells harboring the platform chromosome (Figure 3). Examples of site-specific cassettes that are targeted to the platform chromosome using either mouse rDNA or mouse major repeat DNA include the SV40-attP-hygro cassette and a red fluorescent protein (RFP) gene flanked by loxP sites (Cre/lox, see, *e.g.*, U.S. Patent No. 4,959,317 and description herein). After transfection and integration of the site-specific cassette, homologous recombination events onto the platform chromosome are subcloned and identified by FACS (e.g. screen and single cell subclone via expression of resistance or fluorescent marker) and PCR analysis.

For example, a vector can be constructed containing regions of the mouse rDNA locus flanking a gene cassette containing the SV40 early reporter-bacteriophage lambda attP site-hygromycin selectable marker (see Figure 4 and described below). The use of the bacteriophage lambda attP site for lambda integrase-mediated site-specific recombination is described below. Homologous recombination event of the SV40-attP-hygro cassette onto the platform chromosome was identified using PCR primers that detect the homologous recombination and further confirmed by FISH analysis. After identifying subcloned colonies containing the platform chromosome with a single site-specific recombination site, cells carrying the platform chromosome with a single site-specific

recombination site can now be engineered with site-specific recombinases (e.g. lambda INT, Cre) for integrating a target gene expression vector.

C. Targeting a red fluorescent protein (RFP) gene flanked by loxP sites onto 5B11.12 platform

As another example, while loxP recombination sites could have been introduced onto the *ACes* during *de novo* biosynthesis, it was thought that this might result in multiple segments of the *ACes* containing a high number of loxP sites, potentially leading to instability upon *Cre*-mediated recombination. A gene targeting approach was therefore devised to introduce a more limited number of loxP recombination sites into a locus of the 5B11-12 *ACes* containing introduced and possibly co-amplified endogenous rDNA sequences. Although there are more than 200 copies of rDNA genes in the haploid mouse genome distributed amongst 5-11 chromosomes (depending on strain), rDNA sequences were chosen as the target on the *ACes* since they represent a less frequent target than that of the satellite repeat sequences. Moreover, having observed much stronger pWEPuro9K hybridization to the 5B11-12 *ACes* than to other LMTK$^-$ chromosomes and in light of the observation that the transcribed spacer sequences within the rDNA may be less conserved than the rRNA coding regions, it was contemplated that a targeting vector based on the rDNA gene segment in pWEPuro9K would have a higher probability of targeting to the *ACes* rather than to other LMTK$^-$ chromosomes. Accordingly, a targeting vector, pBSFKLoxDsRedLox, was designed and constructed based on the rDNA sequences contained in pWEPuro9K.

The plasmid pBSFKLoxDsRedLox was generated in 4 steps. First, the *Not*I rDNA insert of pWEPuro9K (Figure 2) was inserted into pBS SK- (Stratagene) giving rise to pBSFK. Second, a loxP polylinker cassette was

generated by PCR amplification of pNEB193 (SEQ ID NO:32; New England Biolabs) using primers complementary to the M13 forward and reverse priming sites at their 3'end and a 34 bp 5' extension comprising a LoxP site. This cassette was reinserted into pNEB193 generating p193LoxMCSLox. Third, the DsRed gene from pDsRed1-N1 (SEQ ID NO:29; Clontech) was then cloned into the polylinker between the loxP sites generating p193LoxDsRedLox. Fourth, a fragment consisting of the DsRed gene flanked by loxP sites was cloned into a unique *Nde*I within the rDNA insert of pBSFK generating pBSFKLoxDsRedLox.

A gel purified 11 Kb *Pml*I /*Eco*RV fragment of pBSFKLoxDsRedLox was used for transfection. To detect targeted integration, PCR primers were designed from rDNA sequences within the 5' *Not*I-*Pml*I fragment of pWEPuro9K that is not present on the targeting fragment (5'primer) and sequence within the LoxDsRedLox cassette (3' primer). If the targeting DNA integrated correctly within the rDNA sequences, PCR amplification using these primers would give rise to a 2.3 Kb band. PCR reactions containing 1-4 μl of genomic DNA were carried out according to the MasterTaq protocol (Eppendorf), using murine rDNA 5' primer (5'-CGGACAATGCGGTTGTGCGT-3'; SEQ ID NO:72) and DsRed 3'primer (5'GGCCCCGTAATGCAGAAGAA-3'; SEQ ID NO:73) and PCR products were analyzed by agarose gel electrophoresis.

1.5×10^6 5B11-12 LMTK$^-$ cells were transfected with 2 μg of the pBSFKLoxDsRedLox targeting DNA described above using Lipofectamine Plus (Invitrogen). For flow sorting, harvested cells were suspended in medium and applied to the Becton Dickinson Vantage SE cell sorter, equipped with 488 nm lasers for excitation and 585/42 bandpass filter for optimum detection of RFP fluorescence. Cells were sorted using dPBS as sheath buffer. Negative control parental 5B11-12 cells and a positive control LMTK$^-$ cell line stably transfected with DsRed were used to

establish the selection gates. The RFP positive gated populations were recovered, diluted in medium supplemented with 1X penicillin-streptomycin (Invitrogen), then plated and cultured as previously described. After 4 rounds of enrichment, the percentage of RFP positive cells reached levels of 50% or higher. DNA from populations was analyzed by PCR for evidence of targeted integration. Ultimately, single cell subclones were established from positive pools and were analyzed by PCR and PCR-positive clones confirmed by FISH as described below. DNA was purified from pools or single cell clones using previously described methods set forth in Lahm et al., Transgenic Res., 1998; 7:131-134, or in some cases using a Wizard Genomic DNA purification kit (Promega). For FISH analysis, a biotinylated DsRed gene probe was generated by PCR using DsRed specific primers and biotin-labeled dUTP (5′ RFP primer: 5′-GGTTTAAAGTGCGCTCCTCCAAGAACGTCATC-3′, SEQ ID NO:74; and 3′ RFP primer: 5′AGATCTAGAGCCGCCGCTACAGGAACAGGTGGTGGCGGCC-3′; SEQ ID NO:75). To maximize the signal intensity of the DsRed probe, Tyramide amplification was carried out according to the manufacturers protocols (NEN).

The process of testing the feasibility of a more general targeting strategy that would not rely on enrichment *via* drug selection of stably transfected clones can be summarized as follows. A red fluorescent protein gene (RFP; encoded by the DsRed gene) was inserted between the loxP sites of the targeting vector to form pBSFKLoxDsRedLox. After transfection with PBSFKLoxDsRedLox, sequential rounds of high speed flow sorting and expansion of sorted cells in culture could then be used to enrich for stable transformants expressing RFP. In the event of targeted integration, PCR screening with primers that amplify from a spacer region within the segment of the 45s pre-rRNA gene in pWEPuro9K to a specific

anchor sequence within the DsRed gene in the targeting cassette would give rise to a diagnostic 2.3 Kb band. However, as rDNA clusters are found on several chromosomes, confirmation of targeting to an *ACes* would require fluorescence in situ hybridization (FISH) analysis. Finally, the flanking of the DsRed gene by loxP sites would allow for its removal and subsequent replacement with other genes of interest.

After transfection of the targeting sequence into 5B11-12 cells, enrichment for targeted clones was carried out using a combination of flow cytometry to detect red-fluorescing cells and PCR screening. Ultimately 17 single cell subclones were identified as potential targeted clones by PCR and of these 16 were found by FISH to contain the DsRed integration event into the *ACes*. These subclones are referred to herein as D11-C4, D11-C12, D11-H3, C9-C9, C9-B9, C9-F4, C9-H8, C9-F2, C9-G8, C9-B6, C9-G3, C9-E12, C9-A11, C11-E3, C11-A9 and C11-H4. PCR analysis of genomic DNA isolated from the D11-C4 subclone gave rise to a 2.3 Kb band, indicative of a targeted integration into an rDNA locus. Further analysis of the subclone by FISH analysis with a DsRed gene probe demonstrated integration of the LoxDsRedLox targeting cassette on the *ACes* co-localizing with one of the regions of rDNA staining seen on the 5B11-12 *ACes*, consistent with a targeted integration into an rDNA locus of the *ACes*, while integrations on other chromosomes were not observed. Since transfected cells were maintained as heterogeneous populations through several cycles of sorting and replating it was not possible to estimate the frequency of targeted events. In most mammalian cell lines the frequency of gene targeting via homologous recombination is roughly 10^{-5}-10^{-7} treated cells. Despite the low frequency of these events in mammalian cells, it is clear that an RFP expression based screening paradigm, coupled with PCR analysis, can effectively detect and enrich for such infrequent events in a large

population. In instances where drug selection is not possible or not desirable, such a system may provide a useful alternative. It was also verified that the modified *ACes* in subclone D11-C4 could be purified by flow cytometry. The results indicate that the flow karyogram of the D11-C4 subclone was unaltered from that of the 5B11-12 cell line. Thus, the D11-C4 *ACes* can be purified in high yield from native chromosomes of the host cell line.

D. Reduction of LoxP on *ACes* to a single site.

The strong hybridization signal detected by FISH on the *ACes* using the DsRed gene probe suggests that several copies of the targeting cassette may be present on the *ACes* in the D11-C4 line. This also suggests that multiple rDNA genes have been correctly targeted.

Accordingly, in certain embodiments where necessary, the number of loxP sites on the *ACes* can be reduced to a single site by *in situ* treatment with *Cre* recombinase, provided that the sites are co-linear. Such a process is described for multiple loxP-flanked integrations on a native mouse chromosome (Garrick et al., Nature Genet., 1998, Jan;18(1):56-59). Reduction to a single loxP site on the D11-C4 *ACes* would result in the loss of the DsRed gene, forming the basis of a useful screen for this event.

For this purpose, a Cre expression plasmid pCX-Cre/GFP III has been generated by first deleting the EcoRI fragment of pCX-eGFP (SEQ ID NO:71) containing the eGFP coding sequence and replacing it with that of a PCR amplified Cre recombinase coding sequence (SEQ ID NO:58), generating pCX-Cre. Next, the AseI/SspI fragment of pD2eGFP-N1 (containing the CMV promoter driving the D2EGFP gene with SV40 polyA signal; Clontech; SEQ ID NO:87) was inserted into the filled HindIII site of pCX-Cre, generating pCX-Cre\GFP III. Control plasmid pCX-CreRev\GFP

III was generated in similar fashion except that the Cre recombinase coding sequence was inserted in the antisense orientation. LMTK⁻ cell line D11-C4 (containing first generation platform *ACes* with multiple loxP-DsRED sites) and 5B11-12 cell line (containing *ACes* with no loxP-DsRED sites) are maintained in culture as described above. D11C4 cells are transfected with 2 μg of plasmid pCX-Cre\GFP III or 2 μg pCX-CreRev\GFP III using Lipofectamine (Invitrogen) as previously described.

Forty-eight to seventy-two hours after transfection, transfected D11-C4 cells are harvested and GFP positive cells are sorted by cell cytometry using a FACSta Vantage cell sorter (Beckton-Dickinson) as follows: All D11-C4 cells transfected with pCX-Cre\GFP III or control plasmid pCX-CreRev\GFP III that exhibit GFP fluorescent higher than the gate level established by untransfected cells are collected and placed in culture a further 7-14 days. After 7-14 days the initial D11-C4 cells are harvested and analyzed by cell cytometry as follows: Untransfected D11-C4 cells are used to establish the gate that defines the RFP positive population, while 5B11-12 cells are used to set the RFP negative gate. The GFP positive population of D11-C4 transfected with pCX-Cre\GFP III should show decreased red fluorescence compared to pCX-CreRev\GFP III transfected or untransfected control D11-C4 cells. The cells exhibiting greatly decreased or no RFP expression are collected and single cell clones subsequently established. These clones will be expanded and analyzed by fluorescence *in-situ* hybridization and Southern blotting to confirm the removal of loxP-DsRed gene copies.

EXAMPLE 3

Construction of targeting vector and transfection into LMtk- cells for the generation of platform chromosomes containing multiple site-specific recombination sites

An example of a selectable marker system for the creation of a chromosome-based platform is shown in Figure 4. This system includes a vector containing the SV40 early promoter immediately followed by (1) a 282 base pair (bp) sequence containing the bacteriophage lambda attP site and (2) the puromycin resistance marker. Initially a *Pvu*II/*Stu*I fragment containing the SV40 early promoter from plasmid pPUR (Clontech Laboratories, Inc., Palo Alto, CA; Seq ID No. 30) was subcloned into the *EcoR*I/CRI site of pNEB193 (a PUC19 derivative obtained from New England Biolabs, Beverly, MA; SEQ ID No. 32) generating the plasmid pSV40193. The only differences between pUC19 and pNEB193 are in the polylinker region. A unique AscI site (GGCGCGCC) is located between the *BamH*I site and the SmaI site, a unique PacI site (TTAATTAA) is located between the *BamH*I site and the *Xba*I site and a unique PmeI site (GTTTAAAC) is located between the *Pst*I site and the *Sa/*I site.

The attP site was PCR amplified from lambda genome (GenBank Accession # NC 001416) using the following primers:

attPUP: CCTTGCGCTAATGCTCTGTTACAGG SEQ ID No. 1

attPDWN: CAGAGGCAGGGAGTGGGACAAAATTG SEQ ID No. 2

After amplification and purification of the resulting fragment, the attP site was cloned into the *Sma*I site of pSV40193 and the orientation of the attP site was determined by DNA sequence analysis (plasmid pSV40193attP). The gene encoding puromycin resistance (Puro) was isolated by digesting the plasmid pPUR (Clontech Laboratories, Inc. Palo Alto, CA) with *Age*I/*BamH*I followed by filling in the overhangs with Klenow and subsequently cloned into the *Asc*I site downstream of the attP site of pSV40193attP generating the plasmid pSV40193attPsensePUR (Figure 4; SEQ ID NO:113)).

The plasmid pSV40193attPsensePUR was digested with *Sca*I and co-transfected with the plasmid pFK161 (SEQ ID NO: 118) into mouse LMtk- cells and platform artificial chromosomes were identified and isolated as described above. The process for generating this exemplary platform *ACes* containing multiple site-specific recombination sites is summarized in Figure 5. One platform *ACes* resulting from this experiment is designated B19-18. This platform *ACes* chromosome may subsequently be engineered to contain target gene expression nucleic acids using the lambda integrase mediated site-specific recombination system as described herein in Example 7 and 8.

EXAMPLE 4

Lambda integrase mediated site-specific recombination of a RFP expressing vector onto artificial chromosomes

In this example, a vector expressing the red fluorescent protein (RFP) was produced and recombined into the attP site residing on an artificial chromosome within LMTK- cells. This recombination is depicted in Figure 7.

A. Construction of expression vectors containing wildtype and mutant lambda integrase

Mutations at the glutamic acid at position 174 in the lambda integrase protein relaxes the requirement for the accessory protein IHF during recombination and DNA supercoiling *in vitro* (see, Miller *et al.* (1980) *Cell 20*:721-729; Lange-Gustafson *et al.* (1984) *J. Biol. Chem. 259*:12724-12732). Mutations at this site promote attP, attB intramolecular recombination in mammalian cells (Lorbach *et al.* (2000) *J. Mol. Biol 296*:1175-1181).

To construct nucleic acid encoding the mutant, lambda integrase was PCR amplified from bacteriophage lambda DNA (cI857 *ind Sam* 7; New England Biolabs) using the following primers:

Lamint1 (SEQ ID No. 3)

TTCGAATTCATGGGAAGAAGGCGAAGTCATGAGCG)
Lamint2 (SEQ ID No. 4)
(TTCGAATTCTTATTTGATTTCAATTTTGTCCCAC).

The resulting PCR product was digested with *Eco*R I and cloned into the *Eco*R I site of pUC19. Lambda integrase was mutated at amino acid position 174 using QuikChange Site-Directed Mutagenesis Kit (Stratagene) and the following oligos (generating a glutamic acid to arginine change at position 174):

LambdaINTE174R
(SEQ ID No. 6)
(CGCGCAGCAAAATCTAGAGTAAGGAGATCAAGACTTACGGCTGACG),
LamintR174rev (SEQ ID No. 7)
(CGTCAGCCGTAAGTCTTGATCTCCTTACTCTAGATTTTGCTGCGCG).

The resulting site directed mutant was confirmed by sequence analysis. The wildtype and mutant lambda genes were cloned into the *Eco*R I site of pCX creating pCX-LamInt (SEQ ID NO: 127) and pCXLamIntR (Figure 8; SEQ ID NO: 112).

The plasmid pCX (SEQ ID No. 70) was derived from plasmid pCXeGFP (SEQ ID No. 71). Excision of the EcoRI fragment containing the eGFP marker generated pCX. To generate plasmid pCXLamINTR (SEQ ID NO: 112) an EcoRI fragment containing the lambda integrase E174R (SEQ ID No. 37) mutation was cloned into the *Eco*RI site of pCX, and to generate plasmid pCX-LamINT, an *Eco*RI fragment containing the wild-type lambda integrase was cloned into the *Eco*RI site of pCX.

B. Construction of integration vector containing attB and DsRed

The plasmid pDsRedN1 (Clontech Laboratories, Palo Alto, CA; SEQ ID No. 29) was digested with *Hpa* I and ligated to the following annealed oligos:

attB1 (SEQ ID No. 8)

(TGAAGCCTGCTTTTTTATACTAACTTGAGCGAA)

attB2 (SEQ ID No. 9)

(TTCGCTCAAGTTAGTATAAAAAAGCAGGCTTCA)

The resulting vector (pDsRedN1-attB) was confirmed by PCR and sequence analysis.

C. Transfection into LMtk- cells

LM(tk-) cells containing the Prototype A *ACes* (L1-18; Chromos Molecular Systems Inc., Burnaby, BC Canada) were co-transfected with pDsRedN1 or pDsRedN1-attB and either pCXLamInt (SEQ ID NO: 127) or pCXLamIntR (SEQ ID NO: 112) using Lipofectamine Plus Reagent (LifeTechnologies, Gaithersburg, MD). The transfected cells were grown in DMEM (LifeTechnologies, Gaithersburg, MD) with 10% FBS (CanSera) and G418 (CalBiochem) at a concentration of 1 mg/ml.

D. Enrichment by cell sorting

The transfected cells were sorted using a FACs Vantage SE cell sorter (Becton Dickenson) to enrich for cells expressing DsRed. The cells were excited with a 488 nm Argon laser at 200 watts and cells fluorescing in the 585/42 detection channel were collected. The sorted cells were returned to growth medium for recovery and expansion. After three successive enrichments for cells expressing DsRed, single cell sorting into 96 well plates was performed using the same parameters. Duplicate plates of the single cell clones were made for PCR analysis.

E. PCR analysis of single cell clones

Pools of cells from each row and column of the 96 well plate were used for DNA isolation. DNA was prepared using a Wizard Genomic DNA purification kit (Promega Inc, Madison, WI). Nested PCR analysis on the DNA pools was performed to confirm the site-specific recombination event using the following primer sets:

attPdwn2 (SEQ ID No. 10)
(TCTTCTCGGGCATAAGTCGGACACC)

CMVen (SEQ ID No. 11)
(CTCACGGGGATTTCCAAGTCTCCAC)

followed by:

attPdwn (SEQ ID No. 12)
(CAGAGGCAGGGAGTGGGACAAAATTG)

CMVen2 (SEQ ID No. 13)
(CAACTCCGCCCCATTGACGCAAATG).

The resulting PCR reactions were analyzed by gel electrophoresis and the potential individual clones containing the site-specific recombination event were identified by combining the PCR results of all of the pooled rows and columns for each 96 well plate. The individual clones were then further analyzed by PCR using the following primers that flank the recombination junction. L1for and F1rev flank the attR junction whereas REDfor and L2rev flank the attL junction (see Figure 7):

L1for (SEQ ID No. 14)
AGTATCGCCGAACGATTAGCTCTTCA

F1rev (SEQ ID No. 15)
GCCGATTTCGGCCTATTGGTTAAA

REDfor (SEQ ID No. 16)
CCGCCGACATCCCCGACTACAAGAA

L2rev (SEQ ID No. 17)
TTCCTTCGAAGGGGATCCGCCTACC.

F. Sequence analysis of recombination junctions

PCR products spanning the recombination junction were Topo-cloned into pcDNA3.1D/V5His (Invitrogen Inc., San Diego, CA) and then sequenced by cycle-sequencing. The clones were confirmed to have the correct *att*R and *att*L junctions by cycle sequencing.

G. Fluorescent In Situ Hybridization (FISH)

The cell lines containing the correct recombination junction sequence were further analyzed by fluorescent *in situ* hybridization (FISH)

by probing with the DsRed coding region labeled with biotin and visualizing with the Tyramide Signal Amplification system (TSA; NEN Life Science Products). The results indicate that the RFP sequence is present on the *ACes*.

H. Southern analysis

Genomic DNA was harvested from the cell lines containing an *ACes* with the correct recombinant event and digested with *Eco*R I. The digested DNAs were separated on a 0.7% agarose gel, transferred and fixed to a nylon membrane and probed with RFP coding sequences. The result showed that there is an integrated copy of RFP coding sequence in each clone.

EXAMPLE 5

Delivery of a second gene encoding GFP onto the RFP platform *ACes*

A. Construction of integration vector containing attB and GFP (pD2eGFPIresPuroattB).

The plasmid pIRESpuro2 (Clontech, Palo Alto, CA; SEQ ID NO: 88) was digested with *Eco*RI and *Not*I then ligated to the D2eGFP *Eco*RI-*Not*I fragment from pD2eGFP-N1 (Clontech, Palo Alto, CA) to create pD2eGFPIresPuro2. Subsequently, oligos encoding the attB site were annealed and ligated into the *Nru*I site of pD2eGFPIresPuro2 to create pD2eGFPIresPuroattB. The orientation of attB in the *Nru*I site was determined by PCR.

B. Transfection of LMtk- cells

The LMtk- cells containing the RFP platform *ACes* produced in Example 4, which has multiple attP sites, were co-transfected with pCXLamIntR and pD2eGFPIresPuroattB using LipofectAMINE PLUS reagent. Five μg of each vector was placed into a tube containing 750 μl of DMEM (Dulbecco's modified Eagles Medium). Twenty μl of the Plus reagent was added to the DNA and incubated at room temperature for 15

minutes. A mixture of 30 μl of lipofectamine and 750 μl DMEM was added to the DNA mixture and incubated an additional 15 minutes at room temperature. The DNA mixture was then added dropwise to approximately 3 million cells attached to a 10cm dish in 5 mls of DMEM. The cells were incubated 4 hours (37°C, 5% CO_2) with the DNA-lipid mixture, after which DMEM with 20% fetal bovine serum was added to the dishes to bring the culture medium to 10% fetal bovine serum. The dishes were incubated at 37°C with 5% CO_2.

Plasmid pD2eGFPIresPuroattB has a puromycin gene transcriptionally linked to the GFP gene *via* an IRES element. Two days after the transfection the cells were placed in medium containing puromycin at 4μg/ml to select for cells containing the pD2eGFPIresPuroattB plasmid integrated into the genome. Twenty-three clones were isolated after 17 days of selection with puromycin. These clones were expanded and then analyzed for the presence of the GFP gene on the *ACes* by 2-color (RFP/biotin & GFP/digoxigenin) TSA-FISH (NEN) according to the manufacturers protocol. Sixteen of the 23 clones produced a positive FISH signal on the *ACes* with a GFP probe.

EXAMPLE 6

Delivery Of *ACes* Into human Mesenchymal Stem Cells (hMSC)

A. Transfection

Transfection conditions for the most efficient delivery of the *ACes* into hMSCs (Cambrex BioWhittaker Product Code PT-2501, lot# F0658, East Rutherford, New Jersey) were assayed using LipofectAMINE PLUS and Superfect. One million prototype B *ACes*, which is a murine derived 60Mb *ACes* having primarily murine pericentric heterochromatin, and carrying a "payload" containing a hygromycin B selectable marker gene and a *lac*Z reporter gene (see , Telenius et al., 1999, Chrom. Res., 7:3-7; and Kereso et al., 1996, Chrom. Res., 4:226-239; each of which is

incorporated herein by reference in its entirety), were combined with 1-12 μl of the transfection agent. In the case of LipofectAMINE PLUS, the PLUS reagent was combined with the *ACes* for 15 minutes followed by LipofectAMINE for a further 15 minutes. Superfect was complexed for 10 minutes at a ratio of 2μl Superfect per 1 million *ACes*. The *ACes*/transfection agent complex was then applied to 0.5 million recipient cells and the transfection was allowed to proceed according to the manufacturer's protocol. Percent transfected cells was determined on a FACS Vantage flow cytometer with argon laser tuned to 488 nm at 200mW and FITC fluorescence collected through a standard FITC 530/30 nm band pass filter. After 24 hours, IdUrd labeled *ACes* were delivered to human MSCs in the range of 30-50%, varying with transfection agent and dose. *ACes* delivery curves were generated from data collected in experiments that varyied the dose of the transfection reagents. Dose response curves of Superfect and LipofectAMINE PLUS, showing delivery of *ACes* into recipient hMSCs cells, were prepared, measured by transfer of IdUrd labeled *ACes* and detected by flow cytometry. Superfect shows maximum delivery in the range of 30-50% at doses greater than 2 μl per million *ACes*. LipofectAMINE PLUS has a 42-48% delivery peak around 5-8 μl per million *ACes*. These dose curves were then correlated with toxicity data to determine the transfection conditions that will allow for highest potential transfection efficiency. Toxicity was determined by a modified plating efficiency assay (de Jong et al., 2001, Chrom. Research, 9:475-485). The population's normalized plating efficiency (at maximum % delivery doses) was in the range of 0.2 - 0.4 for Superfect and 0.5 - 0.6 with LipofectAMINE PLUS.

Due to the transfected population consisting of mixed cell types, flow cytometry allowed for the assessment of *ACes* delivery into each sub-population and the purification of the target population. Flow profiles

showing forward scatter (cell size) and side scatter (internal cell granularity) revealed three distinct hMSC populations that were gated into three regions: R3 (small cell region), R4 (medium cell region), R5 (large cell region). Transfection conditions were further optimized by re-analyzing delivery curves and assessing the differences in delivery to each sub-population. Dose response curves of Superfect and LipofectAMINE were prepared showing % delivery to each sub-population represented by the gating on basis of cell size and granularity properties of the mixed population. Three distinct hMSC populations were gated and % delivery dose curves generated. Using Superfect and LipofectAMINE PLUS the overall % delivery increased with cell size (80-90% delivery in large cells). LipofectAMINE PLUS at high doses (8-12 μl per 1 million *ACes*) shows an increase in the overall proportion of chromosome transfer to the small population (10-20%). This suggests an advantage to using this transfection agent if the small-undifferentiated cell population is the desired target host cell.

B. Expression from Genes on *ACes* IN hMSCs

Following the delivery screening process conducted in section (A) above, the most promising results were subjected to further analyses to monitor expression and verify the presence of structurally intact *ACes*. The transfection conditions employed for these experiments were exactly the same as those that had been used during the screening process. Short-term expression was monitored by transfecting hMSCs with *ACes* containing a RFP gene (red fluorescent protein) set forth in Example 2C as "D11C4". The unselected population was harvested at 72-96 hours post transfection and % positive fluorescent cells measured by flow cytometry. RFP expression was in the range of 1-20%.

Long term-gene expression was assayed by selecting for hygromycin B resistant cells over a period of 7-10 days. Cytogenetic

analysis was done to detect presence of intact *ACes* by Fluorescent *In Situ* hybridization (FISH), where metaphase chromosomes were hybridized to a mouse major satellite-DNA probe (targeting murine pericentric heterochromatin) and a lambda probe (hybridizing to the *lac*Z gene). The human mesenchymal transfected culture could not undergo standard sub-cloning as diffuse colonies form with limited doublings available for expansion. Cytogenetic analysis was performed on the entire population, sampling over a period of 3-10 days post-transfection. The hygromycin resistant population was then blocked in mitosis with colchicine and analyzed for presence of intact *ACes* by FISH. Preliminary FISH results show approximately 2-8% of the hMSC-transfected population had an intact *ACes*. This compared to rat skeletal muscle myoblast clones, which were in the range of 60-95%. To increase the % of intact *ACes* in the hMSC-transfected population an enrichment step can be utilized as described in Example 2C.

C. Differentiation of The hMSCs

In initial experiments where transfected hMSCs cells have been induced to differentiate into adipose or osteocytes, the results indicate that the transfected cells appear to be differentiating at a rate comparable to the untransfected controls and the cultures are lineage specific as tested by microscopic examination, FISH, Oil Red O staining (adipocyte assay), and calcium secretion (osteocyte assay).

Accordingly, these results indicate that the artificial chromosomes (*ACes*) provided herein can be successfully transferred into hMSC target cells. Targeting MSCs (such as hMSCs) permits gene transfer into cells in an undifferentiated state where the cells are easier to expand and purify. The genetically modified cells can then be differentiated *in vitro* or injected into a site *in vivo* where the microenvironment will induce transformation into specific cell lineages.

EXAMPLE 7

Delivery of a Promoterless Marker Gene to a Platform *ACes*

Platform *ACes* containing pSV40attPsensePURO (Figure 4) were constructed as set forth in Examples 3 and 4.

A. Construction of Targeting Vectors.

The base vector p18attBZeo (3166bp; SEQ ID NO: 114) was constructed by ligating the 1067bp *Hin*dIII-*Ssp*I fragment containing attBZeo, obtained from pLITattBZeo (SEQ ID NO:91), into pUC18 (SEQ ID NO: 122) digested with *Hin*dIII and *Ssp*I.

1. p18attBZEO-eGFP (6119bp; SEQ ID NO: 126) was constructed by inserting the 2977bp *Spe*I-*Hin*dIII fragment from pCXeGFP (SEQ ID NO:71; Okabe, *et al.* (1997) *FEBS Lett* 407:313-319) containing the eGFP gene into p18attBZeo (SEQ ID NO: 114) digested with *Hin*dIII and *Xba*I.

2. p18attBZEO-5'6XHS4eGFP (Figure 10; 7631bp; SEQ ID NO: 116) was constructed by ligating the 4465bp *Hin*dIII fragment from pCXeGFPattB(6XHS4)2 (SEQ ID NO: 123), which contains the eGFP gene under the regulation of the chicken beta actin promoter, 6 copies of the HS4 core element located 5' of the chicken beta actin promoter and the polyadenylation signal, into the *Hin*dIII site of p18attBZeo (SEQ ID NO: 114).

3. p18attBZEO-3'6XHS4eGFP (Figure 11; 7600bp; SEQ ID NO: 115) was created by removing the 5'6XHS4 element from p18attBZeo-(6XHS4)2eGFP (SEQ ID NO: 110). p18attBZeo-(6XHS4)2eGFP was digested with *Eco*RV and *Spe*I, treated with Klenow and religated to form p18attBZeo3'6XHS4eGFP (SEQ ID NO: 115).

4. p18attBZEO-(6XHS4)2eGFP (Figure 12; 9080bp; SEQ ID NO: 110) was created in two steps. First, the *Eco*RI-*Spe*I fragment from pCXeGFPattB(6XHS4)2 (SEQ ID NO: 123), which contains 6 copies of the HS4 core element, was ligated into p18attBZeo (SEQ ID NO: 114)

digested with *Eco*RI and *Xba*I to create p18attBZeo6XHS4 (4615bp; SEQ ID NO: 117). Next, p18attBZeo6XHS4 was digested with *Hin*dIII and ligated to the 4465bp *Hin*dIII fragment from pCXeGFPattB(6XHS4)2 which contains the eGFP gene under the regulation of the chicken beta actin promoter, 6 copies of the HS4 core element located 5' of the chicken beta actin promoter and the polyadenylation signal.

Table 2

Targeting plasmid	No. zeocin resistant clones	No. clones with expected PCR product size	No. clones with correct sequence at recombination junction
p18attBZEO-eGFP	12	12	NT*
p18attBZEO-5'6XHS4eGFP	11	11	NT
p18attBZEO-3'6XHS4eGFP	11	11	NT
p18attBZEO-(6XHS4)2eGFP	9	9	4/4

*NT = not tested

B. Transfection and Selection with Drug.

The mouse cell line containing the 2nd generation platform ACE, B19-38 (constructed as set forth in Example 3), was plated onto four 10cm dishes at approximately 5 million cells per dish. The cells were incubated overnight in DMEM with 10% fetal calf serum at 37°C and 5% CO_2. The following day the cells were transfected with 5μg of each of the 4 vectors listed in Example 7.A. above and 5μg of pCXLamIntR (SEQ ID NO: 112), for a total of 10μg per 10cm dish. Lipofectamine Plus reagent was used to transfect the cells according to the manufacturers protocol. Two days post-transfection zeocin was added to the medium at 500μg/ml. The cells were maintained in selective medium until colonies formed. The colonies were then ring-cloned (see, e.g., McFarland, 2000, Methods Cell Sci, Mar;22(1):63-66).

C. Analysis of Clones (PCR, SEQUENCING).

Genomic DNA was isolated from each of the candidate clones with the Wizard kit (Promega) and following the manufacturers protocol. The following primer set was used to analyze the genomic DNA isolated from the zeocin resistant clones: 5PacSV40 - CTGTTAATTAACTGTGGAATGTGTG TCAGTTAGGGTG (SEQ ID NO:76); Antisense Zeo - TGAACAGGGTCACGTCGTCC (SEQ ID NO:77). PCR amplification with the above primers and genomic DNA from the site-specific integration of any of the 4 zeocin vectors would result in a 673bp PCR product.

As set forth in Table 2, of the 4 zeocin resistant candidate clones thusfar analyzed by PCR, all 4 exhibit the correct sequence for a site-specific integration event.

EXAMPLE 8

Integration of a PCR product by site-specific recombination. In this example a gene is integrated onto the platform *ACes* by site-specific recombination without cloning said gene into a vector.

A. PCR PRIMER DESIGN.

PCR primers are designed to contain an attB site at the 5′ end of one of the primers in the primer set. The remaining primers, which could be one or more than one primer, do not contain an attB site, but are complementary to sequences flanking the gene or genes of interest and any associated regulatory sequences. In first example, 2 primers (one containing an attB site) are used to amplify a selective gene such as puromycin.

In a second example as shown in Figure 13, the primer set includes primers 1 & 2 that amplify the GFP gene without amplification of an upstream promoter. Primer 1 contains the attB site at the 5′ end of the oligo. Primers 3 & 4 are designed to amplify the IRES-blasticidin DNA sequences from the vector pIRESblasticidin. The 5′end of primer 3

contains sequences complementary to the 5' end of primer 2 such that annealing can occur between 5' ends of the two primers.

B. PCR REACTION AND SUBSEQUENT LIGATION TO CREATE CIRCULAR MOLECULES FROM THE PCR PRODUCT

In the first example set forth above in Section A, the two PCR primers are combined with a puromycin DNA template such as pPUR (Clontech), a heat stable DNA polymerase and appropriate conditions for DNA amplification. The resulting PCR product (attB-Puromycin) is then then purified and self-ligated to form a circular molecule.

In the second example set forth above in Section A, amplification of the GFP gene and IRES-blasticidin sequences is accomplished by combining primers 1 & 2 with DNA template pD2eGFP and primers 3 & 4 with template pIRESblasticidin under appropriate conditions to amplify the desired template. After initial amplification of the two products (attB-GFP & IRES-blasticidin) in separate reactions, a second round of amplification using both of the PCR products from the first round of amplification together with primers 1 and 4 amplifies the fusion product attB-GFP-IRES-blasticidin (Figure 13). This technique of using complementary sequences in primer design to create a fusion product is employed in *Saccharomyces cerevisiae* for allele replacement (Erdeniz *et al* (1997) ***Gen Res*** 7:1174-1183). The amplified product is then purified from the PCR reaction mixture by standard methods and ligated to form a circular molecule.

C. INTRODUCTION OF PCR PRODUCT ONTO THE *ACes* USING A RECOMBINASE

The circular PCR product is then be introduced to the platform *ACes* using the bacteriophage lambda integrase E174R. The introduction can be performed *in vivo* by transfecting the pCXLamIntR (SEQ ID NO: 112) vector encoding the lambda integrase mutant E174R together with the circularized PCR product into a cell line containing the platform ACE.

D. SELECTION FOR MARKER GENE

The marker gene (in this case either puromycin, blasticidin or GFP) is used to enrich the population for cells containing the proper integration event. A proper integration event in the second example (Figure 14) juxtaposes a promoter residing on the platform *ACes* 5' to the attB-GFP-IRES-Blasticidin PCR product, allowing for transcription of both GFP and blasticidin. If enrichment is done by drug selection, blasticidin is added to the medium on the transfected cells 24-48 hours post-transfection. Selection is maintained until colonies are formed on the plates. If enrichment is done by cell sorting, cells are sorted 2-4 days post-transfection to enrich for cells expressing the fluorescent marker (GFP in this case).

E. ANALYSIS OF CLONES

Clonal isolates are analyzed by PCR, FISH and sequence analysis to confirm proper integration events.

EXAMPLE 9

Construction of a human platform *ACes* "ACE 0.1"

A. CONSTRUCTION OF THE TARGETING VECTOR pPACrDNA

Genome Systems (IncyteGenomics) was supplied with the primers 5'HETS (GGGCCGAAACGATCTCAACCTATT; SEQ ID NO:78), and 3'HETS (CGCAGCGGCCCTCCTACTC; SEQ ID NO:79), which were used to amplify a 538bp PCR product homologous to nt 9680-10218 of the human rDNA sequences (GenBank Accession No. U13369) and used as a probe to screen a human genomic P1AC (P1 Artificial Chromosome) library constructed in the vector pCYPAC2 (Ioannou *et al.* (1994) *Nat. Genet. 6(1):* 84-89). Genome Systems clone #18720 was isolated in this screen and contains three repeats of human rDNA as assessed by restriction analysis. GS clone #18720, was digested with PmeI, a restriction enzyme unique to a single repeat of the human rDNA (45Kbp),

and then religated to form pPACrDNA (Figure 15). The insert in pPACrDNA was analyzed by restriction digests and sequence analysis of the 5' and 3' termini. The pPACrDNA, rDNA sequences are homologous to Genbank Accession #U13369, containing an insert of about 45 kB comprising a single repeat beginning from the end of one repeat at ~33980 (relative to the Genbank sequence) through the beginning of the next repeat up to approximately 35120 (the repeat offset from that listed in the GenBank file). Thus, the rDNA sequence is just over 1 copy of the repeat extending from 33980 (+/-10bp) to the end of the first repeat (43Kbp) and continuing into the second repeat to bp 35120 (+/-10bp).

B. TRANSFECTION AND *ACes* FORMATION.

Five hundred thousand MSU1.1 cells (Morgan et al., 1991, Exp. Cell Res., Nov;197(1):125-136; provided by Dr. Justin McCormick at Michigan State University) were plated per 6cm plate (3 plates total) and allowed to grow overnight. The cells were 70-80% confluent the following day. One plate was transfected with 15μg pPACrDNA (linearized with *Pme* I) and 2μg pSV40attPsensePuro (linearized with *Sca* I; see Example 3). The remaining plates were controls and were transfected with either 20μg pBS (Stratagene) or 20μg pSV40attBsensePuro (linearized with *Sca* I). All three plates were transfected using a $CaPO_4$ protocol.

C. SELECTION OF PUROMYCIN RESISTANT COLONIES

One day post-transfection the cells were "glycerol shocked" by the addition of PBS medium containing 10% glycerol for 30 seconds. Subsequently, the glycerol was removed and replaced with fresh DMEM. Four days post-transfection selective medium was added. Selective medium contains 1μg/ml puromycin. The transfection plates were maintained at 37°C with 5% CO_2 in selective medium for 2 weeks at which point colonies could be seen on the plate transfected with

pPACrDNA and pSV40attPsensePuro. The colonies were ring-cloned from the plate on day 17 post-selection and expanded in selective medium for analysis. Only two colonies (M2-2d & M2-2b) were able to proliferate in the selective medium after cloning. No colonies were seen on the control plates after 37 days in selective medium.

D. ANALYSIS OF CLONES

FISH analysis was performed on the candidate clones to detect *ACes* formation. Metaphase spreads from the candidate clones were probed in multiple probe combinations. In one experiment, the probes used were biotin-labeled human alphoid DNA (pPACrDNA) and digoxigenin-labeled mouse major DNA (pFK161) as a negative control. Candidate M2-2d was single cell subcloned by flow sorting and the candidate subclones were reanalyzed by FISH. Subclone 1B1 of M2-2d was determined to be a platform *ACes* and is also designated human Platform ACE 0.1.

EXAMPLE 10

Site-specific integration of a marker gene onto a human platform ACE 0.1

The promoterless delivery method was used to deliver a promoterless blasticidin marker gene onto the human platform *ACes* with excellent results. The human *ACes* platform with a promoterless blasticidin marker gene resulted in 21 of 38 blasticidin resistant clones displaying a PCR product of the expected size from the population co-transfected with pLIT38attBBSRpolyA10 and pCXLamIntR (Figure 8; SEQ ID NOs. 111 and 112). Whereas, the population transfected with pBlueScript resulted in 0 blasticidin resistant colonies.

A. CONSTRUCTION OF pLIT38attB-BSRpolyA10 & pLIT38attB-BSRpolyA2.

The vector pLITMUS 38 (New England Biolabs; U.S. Patent No. 5,691,140; SEQ ID NO: 119) was digested with *Eco*RV and ligated to

two annealed oligomers, which form an attB site (attB1 5'-TGAAGCCTGCTTTTTTATACTAACTTGAGCGAA-3' (SEQ ID NO:8); attB2 5'- TTCGCTCAAGTTAGTATAAAAAAGCAGGCTTCA-3'; SEQ ID NO:9). This ligation reaction resulted in the vector pLIT38attB (SEQ ID NO: 120). The blasticidin resistance gene and SV40 polyA site were PCR amplified with primers: 5BSD (ACCATGAAAACATTTAACATTTCTCAACA; SEQ ID NO:80) and SV40polyA (TTTATTTGTGAAATTTGTGATGCTATTGC; SEQ ID NO:81) using pPAC4 (Frengen, E., *et al.* (2000) Genomics 68 (2), 118-126; GenBank Accession No. U75992) as template. The blasticidin-SV40polyA PCR product was then ligated into pLIT38attB at the *Bam*HI site, which was Klenow treated following digestion with *Bam*HI. pLIT38attB-BSDpolyA10 (SEQ ID NO: 111) and pLIT38attB-BSDpolyA2 (SEQ ID NO: 121) are the two resulting orientations of the PCR product ligated into the vector.

B. TRANSFECTION OF MSU1.1 CELLS CONTAINING HUMAN PLATFORM ACE 0.1.

MSU1.1 cells containing human platform ACE 0.1 (see Example 9) were expanded and plated to five 10cm dishes with 1.3×10^6 cells per dish. The cells were incubated overnight in DMEM with 10% fetal bovine serum, at 37°C and 5% CO_2. The following day the cells were transfected with 5μg of each plasmid as set forth in Table 3, for a total of 10μg of DNA per plate of cells transfected (see Table 3) using ExGen 500 *in vitro* transfection reagent (MBI fermentas, cat. no. R0511). The transfection was performed according to the manufacturers protocol. Cells were incubated at 37°C with 5% CO_2 in DMEM with 10% fetal bovine serum following the transfection.

Table 3

Plate #	Plasmid 1	Plasmid 2	No. Bsd^R Colonies
1	pBS	None	0
2	pCXLamInt	pLIT38attB-BSRpolyA10	16
3	pCXLamIntR	pLIT38attB-BSRpolyA10	40
4	pCXLamInt	pLIT38attB-BSRpolyA2	28
5	pCXLamIntR	pLIT38attB-BSRpolyA2	36

C. SELECTION OF BLASTICIDIN RESISTANT CLONES.

Three days following the transfection the cells were split from a 10 cm dish to two 15cm dishes. The cells were maintained in DMEM with 10% fetal bovine serum for 4 days in the 15 cm dishes. Seven days post-transfection blasticidin was introduced into the medium. Stably transfected cells were selected with 1μg/ml blasticidin. The number of colonies formed on each plate is listed in Table 3. These colonies were ring-cloned and expanded for PCR analysis. Upon expansion in blasticidin containing medium some clones failed to live and therefore do not have corresponding PCR data.

D. PCR ANALYSIS

Thirty-eight of the 40 clones from plate 3 grew after ring-cloning. Genomic DNA was isolated from these clones with the Promega Wizard Genomic cDNA purification kit, digested with *Eco*RI and used as template in a PCR reaction with the following primers: 3BSP – TTAATTTCGGG TATATTTGAGTGGA (SEQ ID NO:82); 5PacSV40 – CTGTTAATTAACTGTGGAA TGTGTGTCAGTTAGGGTG (SEQ ID NO:76). The PCR conditions were as follows. 100ng of genomic DNA was

amplified with 0.5μl Herculase polymerase (Stratagene) in a 50μl reaction that contained 12.5pmole of each primer, 2.5mM of each dNTP, and 1X Herculase buffer (Stratagene). The reactions were placed in a PerkinElmer thermocycler programmed as follows: Initial denaturation at 95°C for 10 minutes; 35 cycles of 94°C for 1minute, 53°C for 1 minute, 72°C for 1 minute, and 72°C for 1 minute; Final extension for 10 minutes at 72°C; and 4°C hold. If pLIT38attB-BSRpolyA10 integrates onto the human platform ACE 0.1 correctly, PCR amplification with the above primers should yield an 804bp product. Twenty-one of the 38 clones from plate 3 produced a PCR product of the expected 804bp size.

EXAMPLE 11

Delivery of a Vector comprising a Promoterless Marker Gene and a gene encoding a therapeutic product to a Platform *ACes*

Platform *ACes* containing pSV40attPsensePURO (Figure 4) were constructed as set forth in Examples 3 and 4.

A. CONSTRUCTION OF DELIVERY VECTORS

1. Erythropoietin cDNA vector, p18EPOcDNA.

The erythropoietin cDNA was PCR amplified from a human cDNA library (E. Perkins *et al.*, 1999, *Proc. Natl. Acad. Sci. USA 96(5)*: 2204-2209) using the following primers: EPO5XBA - TATCTAGAATGGGGGTGC ACGAATGTCCTGCC (SEQ ID NO: 83); EPO3BSI - TACGTACGTCATC TGTCCCCTGTCCTGCAGGC (SEQ ID NO: 84). The cDNA was amplified through two successive rounds of PCR using the following conditions: heat denaturation at 95°C for 3 minutes; 35 cycles of a 30 second denaturation (95°C), 30 seconds of annealing (60°C), and 1 minute extension (72°C); the last cycle is followed by a 7 minute extension at 72°C. BIO-X-ACT (BIOLINE) was used to amplify the erythropoietin cDNA from 2.5ng of the human cDNA library in the first round of amplification. Five μl of the first amplification product was used

as template for the second round of amplification. Two PCR products were produced from the second amplification with Taq polymerase (Eppendorf), each product was cloned into pCR2.1-Topo (Invitrogen) and sequenced. The larger PCR product contained the expected cDNA sequence for erythropoietin. The erythropoietin cDNA was moved from pTopoEPO into p18attBZeo(6XHS4)2eGFP (SEQ ID NO: 110). pTopoEPO was digested with BsiWI and XbaI to release a 588 bp EPO cDNA. BsrGI and BsiWI create compatable ends. The eGFP gene was removed from p18attBZeo(6XHS4)2eGFP by digestion with BsiWI and XbaI, the 8.3 Kbp vector backbone was gel purified and ligated to the 588 bp EPO cDNA to create p18EPOcDNA (SEQ ID NO: 124).

2. Genomic erythropoietin vector, p18genEPO.

The erythropoietin genomic clone was PCR amplified from a human genomic library (Clontech) using the following primers: GENEPO3BSI - CGTACGTCATCTGTCCCCT GTCCTGCA (SEQ ID NO: 85); GENEPO 5XBA -TCTAGAATGGGGGT GCACGGTGAGTACT (SEQ ID NO: 86). The reaction conditions for the amplification were as follows: heat denaturation for 3 minutes (95°C); 30 cycles of a 30 second denaturation (95°C), 30 seconds annealing (from 65°C decreasing 0.5°C per cycle to 50°C), and 3 minutes extension (72°C); 15 cycles of a 30 second denaturation (95°C), 30 seconds annealing (50°C), and 3 minute extension (72°C); the last cycle is followed by a 7 minute extension at 72°C. The erythropoietin genomic PCR product (2147 bp) was gel purified and cloned into pCR2.1Topo to create pTopogenEPO. Sequence analysis revealed 2bp substitutions and insertions in the intronic sequences of the genomic clone of erythropoietin. A partial digest with XbaI and complete digest with BsiWI excised the erythropoietin genomic insert from pTopogenEPO. The resulting 2158 bp genomic erythropoietin fragment was ligated into the 8.3 Kbp fragment resulting from the

digestion of p18attBZeo(6XHS4)2eGFP (SEQ ID NO: 110) with XbaI and BsrGI to create p18genEPO (SEQ ID NO: 125).

B. TRANSFECTION AND SELECTION WITH DRUG

The erythropoietin genomic and cDNA genes were each moved onto the platform *ACes* B19-38 (constructed as set forth in Example 3) by co-transfecting with pCXLamIntR. Control transfections were also performed using pCXLamInt (SEQ ID NO: 127) together with either p18EPOcDNA (SEQ ID NO: 124) or p18genEPO (SEQ ID NO: 125). Lipofectamine Plus was used to transfect the DNA's into B19-38 cells according to the manufacturer's protocol. The cells were placed in selective medium (DMEM with 10% FBS and Zeocin @ 500ug/ml) 48 hours post-transfection and maintained in selective medium for 13 days. Clones were isolated 15 days post-transfection.

C. ANALYSIS OF CLONES (ELISA, PCR)

1. ELISA Assays

Thirty clones were tested for erythropoietin production by an ELISA assay using a monoclonal anti-human erythropoietin antibody (R&D Systems, Catalogue # MAB287), a polyclonal anti-human erythropoietin antibody (R & D Systems, Catalogue # AB-286-NA) and alkaline phosphotase conjugated goat-anti-rabbit IgG (heavy and light chains) (Jackson ImmunoResearch Laboratories, Inc., Catalogue # 111-055-144). The negative control was a Zeocin resistant clone isolated from B19-38 cells transfected with p18attBZeo(6XHS4) (SEQ ID NO: 117; no insert control vector) and pCXLamIntR (SEQ ID NO: 112). The preliminary ELISA assay was executed as follows: 1) Nunc-Immuno Plates (MaxiSorb 96-well, Catalogue # 439454) were coated with 75μl of a 1/200 dilution (in Phosphate buffered Saline, pH 7.4 (PBS), Sigma Catalogue # P-3813) of monoclonal anti-human erythropoietin antibody overnight at 4°C. 2) The following day the plates were washed 3 times with 300μl PBS

containing 0.15% Tween 20 (Sigma, Catalogue # P-9416). 3) The plates were then blocked with 300μl of 1% Bovine Serum Albumin (BSA; Sigma Catalogue # A-7030) in PBS for 1 hour at 37°C. 4) Repeat the washes as in step 2. 5) The clonal supernatants (75μl per clone per well of 96-well plate) were then added to the plate and incubated for 1 hour at 37°C. The clonal supernatant analyzed in the ELISA assay had been maintained on the cells 7 days prior to analysis. 6) Repeat the washes of step 2. 7) Add 75μl of polyclonal anti-human erythropoietin antibody (1/250 dilution in dilution buffer (0.5% BSA, 0.01% Tween 20, 1X PBS, pH 7.4) and incubate 1 hour at 37°C. 8) Repeat washes of step 2. 9) Add 75μl of goat anti-rabbit conjugated alkaline phosphatase diluted 1/4000 in dilution buffer and incubate 1 hour at 37°C. 10) Repeat washes of step 2. 11) Add 75μl substrate, p-nitrophenyl phosphate (Sigma N2640), diluted to 1mg/ml in substrate buffer (0.1 Ethanolamine-HCl (Sigma, Catalogue # E-6133), 5mM $MgCl_2$ (Sigma, Catalogue # M-2393), pH 9.8). Incubate the plates in the dark for 1 hour at room temperature (22°C). 12) Read the absorption at 405nm (reference wavelength 495nm) on an Universal Microplate Reader (Bio-Tek Instruments, Inc., model # ELX800 UV). The erythropoietin standard curve was derived from readings of diluted human recombinant Erythropoietin (Roche, catalogue # 1-120-166; dilution range 125 - 7.8mUnits/ml). From this preliminary assay the 21 clones displaying the highest expression of erythropoietin were analyzed a second time in the same manner using medium supernatants that had been on the clones for 24 hours and a 1:3 dilution therof.

2. PCR Analysis

Genomic DNA was isolated from the 21 clones with the best expression (as assessed by the initial ELISA assay above) as well as the B19-38 cell line and used for PCR analysis. Genomic DNA was isolated using the Wizard genomic DNA purification kit (Promega) according to the

manufacturers protocol. Amplification was performed on 100ng of genomic DNA as template with MasterTaq DNA Polymerase (Eppendorf) and the primer set 5PacSV40 – CTGTTAATTAACTGTGGAATGTGTG TCAGTTAGGGTG (SEQ ID NO: 76) and Antisense Zeo - TGAACAGGGTCACGTCGTCC (SEQ ID NO: 77). The amplification conditions were as follows: heat denaturation for 3 minutes (95°C); 30 cycles of a 30 second denaturation (95°C), 30 seconds annealing (from 65oC decreasing 0.5oC per cycle to 50°C), and 1 minutes extension (72°C); 15 cycles of a 30 second denaturation (95°C), 30 seconds annealing (50°C), and 1 minute extension (72°C); the last cycle is followed by a 10 minute extension at 72°C. PCR products were size separated by gel electrophoresis. Of the 21 clones analyzed 19 produced a PCR product of 650 bp as expected for a site-specific integration event. All nineteen clones were the result of transformations with p19EPOcDNA (5) or p18genEPO (14) and pCXLamIntR (i.e. mutant integrase). The remaining two clones, both of which were the result of transformation with p18genEPO (SEQ ID NO: 125) and pCXLamInt (i.e. wildtype integrase; SEQ ID NO: 127), produced a 400 bp PCR product.

EXAMPLE 12

Preparation of a Transformation Vector Useful for the Induction of Plant Artificial Chromosome Formation

Plant artificial chromosomes (PACs) can be generated by introducing nucleic acid, such as DNA, which can include a targeting DNA, for example rDNA or lambda DNA, into a plant cell, allowing the cell to grow, and then identifying from among the resulting cells those that include a chromosome with a structure that is distinct from that of any chromosome that existed in the cell prior to introduction of the nucleic acid. The structure of a PAC reflects amplification of chromosomal DNA, for example, segmented, repeat region-containing and heterochromatic

structures. It is also possible to select cells that contain structures that are precursors to PACs, for example, chromosomes containing more than one centromere and/or fragments thereof, and culture and/or manipulate them to ultimately generate a PAC within the cell.

In the method of generating PACs, the nucleic acid can be introduced into a variety of plant cells. The nucleic acid can include targeting DNA and/or a plant expressable DNA encoding one or multiple selectable markers (*e.g.*, DNA encoding bialophos (bar) resistance) or scorable markers (*e.g.*, DNA encoding GFP). Examples of targeting DNA include, but are not limited to, *N. tabacum* rDNA intergenic spacer sequence (IGS) and *Arabidopsis* rDNA such as the 18S, 5.8S, 26S rDNA and/or the intergenic spacer sequence. The DNA can be introduced using a variety of methods, including, but not limited to *Agrobacterium*-mediated methods, PEG-mediated DNA uptake and electroporation using, for example, standard procedures according to Hartmann *et al* [(1998) *Plant Molecular Biology 36*:741]. The cell into which such DNA is introduced can be grown under selective conditions and can initially be grown under non-selective conditions and then transferred to selective media. The cells or protoplasts can be placed on plates containing a selection agent to grow, for example, individual calli. Resistant calli can be scored for scorable marker expression. Metaphase spreads of resistant cultures can be prepared, and the metaphase chromosomes examined by FISH analysis using specific probes in order to detect amplification of regions of the chromosomes. Cells that have artificial chromosomes with functioning centromeres or artificial chromosomal intermediate structures, including, but not limited to, dicentric chromosomes, formerly dicentric chromosomes, minichromosomes, heterochromatin structures (e.g. sausage chromosomes), and stable self-replicating artificial chromosomal intermediates as described herein, are

identified and cultured. In particular, the cells containing self-replicating artificial chromosomes are identified.

The DNA introduced into a plant cell for the generation of PACs can be in any form, including in the form of a vector. An exemplary vector for use in methods of generating PACs can be prepared as follows.

For the production of artificial chromosomes, plant transformation vectors, as exemplified by pAgIIa and pAgIIb, containing a selectable marker, a targeting sequence, and a scorable marker were constructed using procedures well known in the art to combine the various fragments. The vectors can be prepared using vector pAg1 as a base vector and inserting the following DNA fragments into pAg1: DNA encoding β-glucoronidase under the control of the nopaline synthase (NOS) promoter fragment and flanked at the 3′ end by the NOS terminator fragment, a fragment of mouse satellite DNA and an *N. tabacum* rDNA intergenic spacer sequence (IGS). In constructing plant transformation vectors, vector pAg2 can also be used as the base vector.

1. **Construction of pAG1**

Vector pAg1 (SEQ. ID. NO: 89) is a derivative of the CAMBIA vector named pCambia 3300 (Center for the Application of Molecular Biology to International Agriculture, i.e., CAMBIA, Canberra, Australia; www.cambia.org), which is a modified version of vector pCambia 1300 to which has been added DNA from the bar gene confering resistance to phosphinothricin. The nucleotide sequence of pCambia 3300 is provided in SEQ. ID. NO: 90. pCambia 3300 also contains a lacZ alpha sequence containing a polylinker region.

pAg1 was constructed by inserting two new functional DNA fragments into the polylinker of pCambia 3300: one sequence containing an attB site and a promoterless zeomycin resistance-encoding DNA flanked at the 3′ end by a SV40 polyA signal sequence, and a second

sequence containing DNA from the hygromycin resistance gene (hygromycin phosphotransferase) confering resistance to hygromycin for selection in plants. Although the zeomycin-SV40 polyA signal fusion is not expected to function in plant cells, it can be activated in mammalian cells by insertion of a functional promoter element into the attB site by site-specific recombination catalyzed by the Lambda att integrase. Thus, the inclusion of the attB-zeomycin sequences allows for evaluation of functionality of plant artificial chromosomes in mammalian cells by activation of the zeomycin resistance-encoding DNA, and provides an att site for further insertion of new DNA sequences into plant artificial chromosomes formed as a result of using pAg1 for plant transformation. The second functional DNA fragment allows for selection of plant cells with hygromycin. Thus, pAg1 contains DNA from the bar gene confering resisance to phosphinothricin, DNA from the hygromycin resistance gene, both resistance-encoding DNAs under the control of a separate cauliflower mosaic virus (CaMV) 35S promoter, and the attB-promoterless zeomycin resistance-encoding DNA.

pAg1 is a binary vector containing *Agrobacterium* right and left T-DNA border sequences for use in *Agrobacterium*-mediated transformation of plant cells or protoplasts with the DNA located between the border sequences. pAg1 also contains the pBR322 Ori for replication in *E.coli.* pAg1 was constructed by ligating *Hind*III/*Pst*I-digested p3300attBZeo with *Hind*III/*Pst*I-digested pBSCaMV35SHyg as follows.

a. Generation of p3300attBZeo

Plasmid pCambia 3300 was digested with *Pst*I/*Ecl*136 II and ligated with *Pst*I/*Stu*I-digested pLITattBZeo (the nucleotide sequence of pLITattBZeo is provided in SEQ. ID. NO: 91), which contains DNA encoding the zeocin resistance gene and an attB Integrase recognition sequence, to generate p3300attBZeo which contains an attB site, a promoterless

zeomycin resistance-encoding DNA flanked at the 3' end by a SV40 polyA signal, and a reconstructed *Pst*I site.

b. Generation of pBSCaMV35SHyg

A DNA fragment containing DNA encoding hygromycin phosphotransferase flanked by the CaMV 35S promoter and the CaMV 35S polyA signal sequence was obtained by PCR amplification of plasmid pCambia 1302 (GenBank Accession No. AF234298 and SEQ. ID. NO: 92). The primers used in the amplification reaction were as follows:

CaMV35SpolyA:
5'-CTGAATTAACGCCGAATTAATTCGGGGGATCTG-3' SEQ. ID. NO: 93
CaMV35Spr:
5'-CTAGAGCAGCTTGCCAACATGGTGGAGCA-3' SEQ. ID. NO: 94

The 2100-bp PCR fragment was ligated with *Eco*RV-digested pBluescript II SK+ (Stratagene, La Jolla, CA, U.S.A.) to generate pBSCaMV35SHyg.

c. Generation of pAg1

To generate pAg1, pBSCaMV35SHyg was digested with *Hind*III/*Pst*I and ligated with *Hind*III/*Pst*I-digested p3300attBZeo. Thus, pAg1 contains the pCambia 3300 backbone with DNA conferring resistance to phophinothricin and hygromycin under the control of separate CaMV 35S promoters, an attB-promoterless zeomycin resistance-encoding DNA recombination cassette and unique sites for adding additional markers, *e.g.*, DNA encoding GFP. The attB site can be used as decribed herein for the addition of new DNA sequences to plant artificial chromosomes, including PACs formed as a result of using the pAg1 vector, or derivatives thereof, in the production of PACs. The attB site provides a convenient site for recombinase-mediated insertion of DNAs containing a homologous att site.

2. pAG2

The vector pAg2 (SEQ. ID. NO: 95) is a derivative of vector pAg1 formed by adding DNA encoding a green fluorescent protein (GFP), under the control of a NOS promoter and flanked at the 3' end by a NOS polyA signal, to pAg1. pAg2 was constructed as follows. A DNA fragment containing the NOS promoter was obtained by digestion of pGEM-T-NOS, or pGEMEasyNOS (SEQ. ID. NO: 96), containing the NOS promoter in the cloning vector pGEM-T-Easy (Promega Biotech, Madison, WI, U.S.A.), with *Xba*I/*Nco*I and was ligated to a *Xba*I/*Nco*I fragment of pCambia 1302 containing DNA encoding GFP (without the CaMV 35S promoter) to generate p1302NOS (SEQ. ID. NO: 97) containing GFP-encoding DNA in operable association with the NOS promoter. Plasmid p1302NOS was digested with *Sma*I/*Bsi*WI to yield a fragment containing the NOS promoter and GFP-encoding DNA. The fragment was ligated with *Pme*I/*Bsi*WI-digested pAg1 to generate pAg2. Thus, pAg2 contains DNA from the bar gene confering resistance to phosphinothricin, DNA conferring resistance to hygromycin, both resistance-encoding DNAs under the control of a cauliflower mosaic virus 35S promoter, DNA encoding kanamycin resistance, a GFP gene under the control of a NOS promoter and the attB-zeomycin resistance-encoding DNA. One of skill in the art will appreciate that other fragments can be used to generate the pAg1 and pAg2 derivatives and that other heterlogous DNA can be incorporated into pAg1 and pAg2 derivatives using methods well known in the art.

3. pAgIIa and pAgIIb transformation vectors

Vectors pAgIIa and pAgIIb were constructed by inserting the following DNA fragments into pAg1: DNA encoding β-glucoronidase, the nopaline synthase terminator fragment, the nopaline synthase (NOS) promoter fragment, a fragment of mouse satellite DNA and an *N. tabacum*

rDNA intergenic spacer sequence (IGS). The construction of pAgIIa and pAgIIb was as follows.

An *N. tabacum* rDNA intergenic spacer (IGS) sequence (SEQ. ID. NO: 98; see also GenBank Accession No. YO8422; see also Borysyuk *et al.* (2000) *Nature Biotechnology 18*:1303-1306; Borysyuk *et al.* (1997) *Plant Mol. Biol.35*:655-660; U.S. Patent Nos. 6,100,092 and 6,355,860) was obtained by PCR amplification of tobacco genomic DNA. The IGS can be used as a targeting sequence by virtue of its homology to tobacco rDNA genes; the sequence is also an amplification promoter sequence in plants. This fragment was amplified using standard PCR conditions (*e.g.*, as described by Promega Biotech, Madison, WI, U.S.A.) from tobacco genomic DNA using the primers shown below:

NTIGS-FI

5'- GTG CTA GCC AAT GTT TAA CAA GAT G- 3' (SEQ ID No. 99) and

NTIGS-RI

5'-ATG TCT TAA AAA AAA AAA CCC AAG TGA C- 3' (SEQ ID No. 100)

Following amplification, the fragment was cloned into pGEM-T Easy to give pIGS-I A fragment of mouse satellite DNA (Msat1 fragment; GenBank Accession No. V00846; and SEQ ID No. 101) was amplified via PCR from pSAT-1 using the following primers:

MSAT-F1

5'- AAT ACC GCG GAA GCT TGA CCT GGA ATA TCG C -3'(SEQ ID No. 102) and

MSAT-Ri

5'-ATA ACC GCG GAG TCC TTC AGT GTG CA T- 3' (SEQ ID No. 103)

This amplification added a *Sac*II and a *Hind*III site at the 5'end and a *Sac*II site at the 3' end of the PCR fragment. This fragment was then cloned into the *Sac*II site in pIGS-1 to give pMIGS-1, providing a eukaryotic

centromere-specific DNA and a convenient DNA sequence for detection via FISH.

A functional marker gene containing a NOS-promoter:GUS:NOS terminator fusion was then constructed containing the NOS promoter (GenBank Accession No. U09365; SEQ ID No. 104), *E. coli* β-glucuronidase coding sequence (from the GUS gene; GenBank Accession No. S69414; and SEQ ID No. 105), and the nopaline synthase terminator sequence (GenBank Accession No. U09365; SEQ ID No. 107). The NOS promoter in pGEM-T-NOS was added to a promoterless GUS gene in pBlueScript (Stratagene, La Jolla, CA, U.S.A.) using *Not*I/*Spe*I to form pNGN-1, which has the NOS promoter in the opposite orientation relative to the GUS gene.

pMIGS-1 was digested with *Not*I/*Spe*I to yield a fragment containing the mouse major satellite DNA and the tobacco IGS which was then added to *Not*I-digested pNGN-1 to yield pNGN-2. The NOS promoter was then re-oriented to provide a functional GUS gene, yielding pNGN-3, by digestion and religation with *Spe*I. Plasmid pNGN-3 was then digested with *Hind*III, and the *Hind*III fragment containing the β-glucuronidase coding sequence and the rDNA intergenic spacer, along with the Msat sequence, was added to pAG-1 to form pAgIIa (SEQ ID NO: 108), using the unique *Hind*III site in pAg1 located near the right T-DNA border of pAg1, within the T-DNA region.

Another plasmid vector, referred to as pAgIIb, was also recovered, which contained the inserted HindIII fragment (SEQ ID NO: 108) in the opposite orientation relative to that observed in pAgIIa. Thus, pAgIIa and pAgIIb differ only in the orientation of the HindIII fragment containing the mouse major satellite sequence, the GUS DNA sequence and the IGS sequence. The nucleotide sequence of pAgIIa is provided in SEQ. ID. NO: 109.

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A eukaryotic chromosome comprising one or a plurality of *att* site(s), wherein:

an *att* site is heterologous to the chromosome; and

an *att* site permits site-directed integration in the presence of lambda integrase.

2. The eukaryotic chromosome of claim 1, wherein the *att* sites are selected from the group consisting of *att*P and *att*B or *att*L and *att*R, or variants thereof.

3. The eukaryotic chromosome of claim 1 that is an artificial chromosome.

4. The eukaryotic chromosome of claim 1 that is an artificial chromosome expression system (*ACes*).

5. The eukaryotic chromosome of claim 4 that is predominantly heterochromatin.

6. The chromosome of claim 1 that is an artificial chromosome that contains no more than about 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% euchromatin.

7. The chromosome of claim 1 that is a plant chromosome.

8. The chromosome of claim 1 that is an animal chromosome.

9. The chromosome of claim 7 that is a plant artificial chromosome.

10. The chromosome of claim 8 that is an animal artificial chromosome.

11. The chromosome of claim 8 that is a mammalian chromosome.

12. The chromosome of claim 11 that is a mammalian artificial chromosome.

13. The chromosome of claim 6 that is an artificial chromosome expression system (*ACes*).

14. A platform artificial chromosome expression system (*ACes*) comprising one or a plurality of sites that participate in recombinase catalyzed recombination.

15. The *ACes* of claim 14 that contains one site.

16. The *ACes* of claim 14 that is predominantly heterochromatin.

17. The *ACes* of claim 14 that contains no more than about 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% euchromatin.

18. The *ACes* of claim 14 that is a plant *ACes*.

19. The *ACes* of claim 14 that is an animal *ACes*.

20. The *ACes* of claim 14 that is selected from a fish, insect, reptile, amphibian, arachnid or a mammalian *ACes*.

21. The *ACes* of claim 14 that is a fish *ACes*.

22. The artificial chromosome expression system (*ACes*) of claim 14, wherein the recombinase and site(s) are from the Cre/lox system of bacteriophage P1, the int/att system of lambda phage, the FLP/FRT system of yeast, the Gin/gix recombinase system of phage Mu, the Cin recombinase system, the Pin recombinase system of *E. coli* and the R/RS system of the pSR1 plasmid, or any combination thereof.

23. A method of introducing heterologous nucleic acid into a chromosome, comprising:

contacting a chromosome of any of claims 1 or 14 with a nucleic acid molecule comprising both the heterologous nucleic acid and a recombination site, in the presence of a recombinase that promotes recombination between the sites in the chromosome and in the nucleic acid molecule.

24. The method of claim 23, wherein the recombinase is selected from the group consisting of Cre, Gin, Cin, Pin, FLP, a phage integrase and R from the pSR1 plasmid.

25. The method of claim 23, wherein the nucleic acid molecule encodes a therapeutic protein, antisense nucleic acid, or comprises an artificial chromosome.

26. The method of claim 25, wherein the nucleic acid molecule comprises a yeast artificial chromosomes (YAC), a bacterial artificial chromosome (BAC) or an insect artificial chromosome (IAC).

27. A combination, comprising, the chromosome of claim 1 and a first vector comprising the cognate recombination site, wherein the cognate recombination site is a site that recombines with the site engineered into the chromosome.

28. The combination of claim 27, further comprising nucleic acid encoding a recombinase, wherein the nucleic acid is on a second vector or on the first vector, or on the *ACes* under an inducible promoter.

29. The combination of claim 28, wherein the recombinase and sites are from the Cre/lox system of bacteriophage P1, the int/att system of lambda phage, the FLP/FRT system of yeast, the Gin/gix recombinase system of phage Mu, the Pin recombinase system of *E. coli* and the R/RS system of the pSR1 plasmid, or any combination thereof.

30. The combination of claim 28, wherein a vector is the plasmid pCXLamIntR.

31. The combination of claim 27, wherein a vector is the plasmid pDsRedN1-attB.

32. A kit, comprising the combination of claim 27 and optionally instructions for introducing heterologous nucleic acid into the chromosome.

33. A method for introducing heterologous nucleic acid into a platform artificial chromosome, comprising:

(a) mixing an artificial chromosome comprising at least a first recombination site and a vector comprising at least a second recombination site and the heterologous nucleic acid;

(b) incubating the resulting mixture in the presence of at least one recombination protein under conditions whereby recombination between the first and second recombination sites is effected, thereby introducing the heterologous nucleic acid into the artificial chromosome.

34. The method of claim 33, wherein the artificial chromosome is an *ACes*.

35. The method of claim 33, wherein said mixing step (a) is conducted in cells ex vivo.

36. The method of claim 33, wherein said mixing step (a) is conducted extracellularly in an in vitro reaction mixture.

37. The method of claim 33, wherein the at least one recombination protein is encoded by a bacteriophage selected from the group consisting of bacteriophage lambda, phi 80, P22, P2, 186, P4 and P1.

38. The method of claim 37, wherein the at least one recombination protein is encoded by bacteriophage lambda, or mutants thereof.

39. The method of claim 33, wherein at least one recombination protein is selected from the group consisting of Int, IHF, Xis and Cre, $\gamma\delta$, Tn3 resolvase, Hin, Gin, Cin and Flp.

40. The method of claim 32, wherein the recombination sites are selected from the group consisting of att and lox P sites.

41. The method of claim 33, wherein the first and/or second recombination site contains at least one mutation that removes one or more stop codons.

42. The method of claim 33, wherein the first and/or second recombination site contains at least one mutation that avoids hairpin formation.

43. The method of claim 33, wherein the first and/or second recombination site comprises at least a first nucleic acid sequence selected from the group consisting of SEQ ID NOs:41-56:

a) RKYCWGCTTTYKTRTACNAASTSGB (m-att) (SEQ ID NO:41);
b) AGCCWGCTTTYKTRTACNAACTSGB (m-attB) (SEQ ID NO:42);
c) GTTCAGCTTTCKTRTACNAACTSGB (m-attR) (SEQ ID NO:43);
d) AGCCWGCTTTCKTRTACNAAGTSGB (m-attL) (SEQ ID NO:44);
e) GTTCAGCTTTYKTRTACNAAGTSGB (m-attP1) (SEQ ID NO:45);
f) AGCCTGCTTTTTTGTACAAACTTGT (attB1) (SEQ ID NO:46);
g) AGCCTGCTTTCTTGTACAAACTTGT (attB2) (SEQ ID NO:47);
h) ACCCAGCTTTCTTGTACAAACTTGT (attB3) (SEQ ID NO:48);
i) GTTCAGCTTTTTTGTACAAACTTGT (attR1) (SEQ ID NO:49);
j) GTTCAGCTTTCTTGTACAAACTTGT (attR2) (SEQ ID NO:50);
k) GTTCAGCTTTCTTGTACAAAGTTGG (attR3) (SEQ ID NO:51);
l) AGCCTGCTTTTTTGTACAAAGTTGG (attL1) (SEQ ID NO:52);
m) AGCCTGCTTTCTTGTACAAAGTTGG (attL2) (SEQ ID NO:53);
n) ACCCAGCTTTCTTGTACAAAGTTGG (attL3) (SEQ ID NO:54);
o) GTTCAGCTTTTTTGTACAAAGTTGG (attP1) (SEQ ID NO:55);
p) GTTCAGCTTTCTTGTACAAAGTTGG (attP2, P3) (SEQ ID NO: 56);

and a corresponding or complementary DNA or RNA sequence, wherein R=A or G, K=G or T/U, Y=C or T/U, W=A or T/U, N=A or C or G or T/U, S=C or G, and B=C or G or T/U; and

the core region does not contain a stop codon in one or more reading frames.

44. The method of claim 33, wherein the first and/or second recombination site comprises at least a first nucleic acid sequence selected from the group consisting of a mutated att recombination site containing at least one mutation that enhances recombinational specificity, a complementary DNA sequence thereto, and an RNA sequence corresponding thereto.

45. The method of claim 33, wherein the vector comprising the second site further encodes at least one selectable marker.

46. The method of claim 45, wherein the marker is a promoterless marker, which, upon recombination is under the control of a promoter and is thereby expressed.

47. The method of claim 46, wherein the first recombination site is attP and is in the sense orientation prior to recombination.

48. The method of claim 46, wherein the selectable marker is selected from the group consisting of an antibiotic resistance gene, and a detectable protein, wherein the detectable protein is chromogenic, fluorescent, or capable of being bound by an antibody and FACs sorted.

49. The method of claim 48, wherein the selectable marker is selected from the group consisting of green fluorescent protein (GFP), red fluorescent protein (RFP), blue fluorescent protein (BFP), and *E. coli* histidinol dehydrogenase (hisD).

50. A cell comprising, the chromosome of claim 1.

51. The cell of claim 50, wherein the cell is a nuclear donor cell.

52. The cell of claim 50, wherein the cell is a stem cell.

53. The stem cell of claim 52, wherein said stem cell is human and is selected from the group consisting of a mesenchymal stem cell, a hematopoietic stem cell, an adult stem cell and an embryonic stem cell.

54. The cell of claim 50, wherein the cell is mammalian.

55. The cell of claim 54, wherein the mammal is selected from the group consisting of humans, primates, cattle, pigs, rabbits, goats, sheep, mice, rats, guinea pigs, hamsters, cats, dogs, and horses.

56. The cell of claim 50, wherein the cell is a plant cell.

57. A cell comprising the platform *ACes* of claim 14.

58. The cell of claim 57, wherein the cell is a nuclear donor cell.

59. The cell of claim 57, wherein the cell is a stem cell.

60. The stem cell of claim 59, wherein said stem cell is human and is selected from the group consisting of a mesenchymal stem cell, a hematopoietic stem cell, an adult stem cell and an embryonic stem cell.

61. A human mesenchymal cell comprising an artificial chromosome.

62. The human mesenchymal cell of claim 61, wherein said artificial chromosome is an *ACes*.

63. The human mesenchymal cell of claim 62, wherein the *ACes* is a platform-*ACes*.

64. A method for introducing heterologous nucleic acid into the mesenchymal cell of claim 63, comprising:

(a) introducing into the cell of claim 63, wherein the platform-*ACes* has a first recombination site, a vector comprising at least a second recombination site and the heterologous nucleic acid;

(b) incubating the resulting mixture in the presence of at least one recombination protein under conditions whereby recombination between the first and second recombination sites is effected, thereby introducing the heterologous nucleic acid into the platform-*ACes* within the mesenchymal cell.

65. A lambda-intR mutein comprising a glutamic acid to arginine change at position 174 of wild-type lambda-intR.

66. The lambda-intR mutein of claim 65, wherein the lambda-intR mutein comprises SEQ ID NO:37.

67. The method of claim 46 wherein the promoterless marker is transcriptionally downstream of the heterologous nucleic acid, wherein the heterologous nucleic acid encodes a heterologous protein, and wherein the expression level of the selectable marker is transcriptionally linked to the expression level of the heterologous protein.

68. The method of claim 67, wherein the selectable marker and the heterologous nucleic acid are transcriptionally linked by the presence of a IRES between them.

69. The method of claim 68, wherein the selectable marker is selected from the group consisting of an antibiotic resistance gene, and a detectable protein, wherein the detectable protein is chromogenic or fluorescent.

70. The method of claim 69, wherein the selectable marker is selected from the group consisting of green fluorescent protein (GFP), red fluorescent protein (RFP), blue fluorescent protein (BFP), and *E. coli* histidinol dehydrogenase.

71. The method of claim 67 further comprising expressing the heterologous protein and isolating the heterologous protein.

72. A method for producing a transgenic animal, comprising introducing a platform-*ACes* into an embryonic cell.

73. The method of claim 72, wherein the embryonic cell is a stem cell.

74. The method of claim 72, wherein the embryonic cell is in an embryo.

75. The method of claim 72, wherein the platform-*ACes* comprises heterologous nucleic acid that encodes a therapeutic product.

76. The method of claim 72, wherein the transgenic animal is a fish, insect, reptile, amphibians, arachnid or mammal.

77. The method of claim 72, wherein the *ACes* is introduced by cell fusion, lipid-mediated transfection by a carrier system, microinjection, microcell fusion, electroporation, microprojectile bombardment or direct DNA transfer.

78. A transgenic animal produced by the method of claim 72.

79. A cell line useful for making a library of *ACes*, comprising a multiplicity of heterologous recombination sites randomly integrated throughout the endogenous chromosomes.

80. A method of making a library of *ACes* comprising random portions of a genome, comprising introducing one or more *ACes* into the cell line of claim 79, under conditions that promote the site-specific chromosomal arm exchange of the *ACes* into, and out of, a multiplicity of the heterologous recombination sites within the cell's chromosomal DNA; and isolating said multiplicity of *ACes*, thereby producing a library of *ACes* whereby multiple *ACes* have different portions of the genome within.

81. A library of cells useful for genomic screening, said library comprising a multiplicity of cells, wherein each cell comprises an *ACes* having a mutually exclusive portion of a chromosomal nucleic acid therein.

82. The library of cells of claim 81, wherein the cells of the library are from a different species than the chromosomal nucleic acid within the *ACes*.

83. A method of making one or more cell lines, comprising

a) integrating into endogenous chromosomal DNA of a selected cell species, a multiplicity of heterologous recombination sites,

b) introducing a multiplicity of *ACes* under conditions that promote the site-specific chromosomal arm exchange of the *ACes* into, and out of, a multiplicity of the heterologous recombination sites integrated within the cell's endogenous chromosomal DNA;

c) isolating said multiplicity of *ACes*, thereby producing a library of *ACes* whereby a multiplicity of *ACes* have mutually exclusive portions of the endogenous chromosomal DNA therein;

d) introducing the isolated multiplicity of *ACes* of step c) into a multiplicity of cells, thereby creating a library of cells;

e) selecting different cells having mutually exclusive *ACes* therein and clonally expanding or differentiating said different cells into clonal cell cultures, thereby creating one or more cell lines.

84. The method of claim 23, wherein the nucleic acid molecule with a recombination site is a PCR product.

85. Method of claim 23 wherein the recombinase is a protein and the recombination event occurs in vitro.

86. The method of claim 33, wherein the vector is a PCR product comprising a second recombination site.

87. The lambda-intR mutein of claim 65, wherein the mutein further comprises an amino acid signal for nuclear localization.

88. The lambda-intR mutein of claim 65, wherein the mutein further comprises an epitope tag for protein purification.

89. A modified iron-induced promoter comprising SEQ ID NO:128.

90. A plasmid or expression cassette comprising the promoter of claim 89.

91. A vector, comprising:

a recognition site for recombination; and

a sequence of nucleotides that targets the vector to an amplifiable region of a chromosome.

92. The vector of claim 91, wherein the amplifiable region comprises heterochromatic nucleic acid.

93. The vector of claim 91, wherein the amplifiable region comprises rDNA.

94. The vector of claim 93, wherein the rDNA comprises an intergenic spacer.

95. The vector of claim 91, further comprising nucleic acid encoding a selectable marker that is not operably associated with any promoter.

96. The vector of claim 91, wherein the chromosome is a mammalian chromosome.

97. The vector of claim 91, wherein the chromosome is a plant chromosome.

98. A cell of claim 57 that is a plant cell, wherein the *ACes* platform is a MAC.

99. The plant cell of claim 98, wherein the MAC comprises transcriptional regulatory sequence of nucleotides derived from plants.

100. The plant cell of claim 99, wherein the regulatory sequence is selected from the group consisting of promoters, terminators, enhancers, silencers and transcription factor binding sites.

101. A cell of claim 57 that is an animal cell, wherein the *ACes* platform is a plant artificial chromosome (PAC).

102. The cell of claim 101 that is a mammalian cell.

103. The cell of claim 98, wherein the MAC comprises transcriptional regulatory sequence of nucleotides derived from plants.

104. The cell of claim 102, wherein the MAC comprises transcriptional regulatory sequence of nucleotides derived from plants.

105. The cell of claim 104, wherein the regulatory sequence is selected from the group consisting of promoters, terminators, enhancers, silencers and transcription factor binding sites.

106. A method, comprising:

introducing a vector of claim 91 into a cell;

growing the cells; and

selecting a cell comprising an artificial chromosome that comprises one or more repeat regions.

107. The method of claim 106, wherein sufficient portion of the vector integrates into a chromosome in the cell to result in amplification of chromosomal DNA.

108. The method of claim 106, wherein the artificial chromsome is an *ACes*.

109. A method for screening, comprising:

contacting a cell comprising a reporter *ACes* with test compounds or known compounds, wherein:

the reporter *ACes* comprises one or a plurality of reporter constructs;

a reporter construct comprises a reporter gene in operative linkage with a regulatory region responsive to test or known compounds; and

detecting any increase or decrease in signal output from the reporter, wherein a change in the signal is indicative of activity of the test or known compound on the regulatory region.

110. The method of claim 109, wherein the reporter is operatively linked to a promoter that controls expression of a gene in a signal transduction pathway, whereby activation or reduction in the signal indicates that the pathway is activated or down-regulated by the test compound.

111. The method of claim 109, wherein the reporter in the construct encodes drug resistance or encodes a fluorescent protein.

112. The method of claim 111, wherein the fluorescent protein is selected from the group consisting of red, green and blue fluorescent proteins.

113. The method of claim 109, wherein the *ACes* comprises a plurality of reporter-linked constructs, each with a different reporter, whereby the pathway(s) affected by the test compounds can be elucidated.

114. The method of claim 109, wherein a reporter is operatively linked to a promoter that is transcriptionally regulated in resopnse to DNA damage, and the test compounds are genotoxicants.

115. The method of claim 114, wherein the DNA damage is induced by apoptosis, necrosis or cell-cycle perturbations.

116. The method of claim 114, wherein unknown compounds are screened to assess whether they are genotoxicants.

117. The method of claim 114, wherein the promoter is a cytochrome P450-profiled promoter.

118. The method of claim 114, wherein the cell is in a transgenic animal and toxicity is assessed in the animal.

119. The method of claim 109, wherein:

the cell is a patient cell sample; the patient has a disease;

the regulatory region is one targeted by a drug or drug regimen; and

the method assesses the effectiveness of a treatment for the disease for the particular patient.

120. The method of claim 119, wherein the cell is a tumor cell.

121. The method of claim 109, wherein the cell is a stem cell or a progenitor cell, whereby expression of the reporter is operatively linked to

a regulatory region exprssed in the cells to thereby identify stem cells or progenitor cell.

122. The method of claim 109, wherein the cell is in an animal; and the method comprises whole-body imaging to monitor expression of the reporter in the animal.

123. A reporter *ACes* comprises one or a plurality of reporter constructs, wherein the reporter construct comprises a reporter gene in operative linkage with a regulatory region responsive to test or known compounds.

Generation of *ACes* for Platform Chromosome Engineering

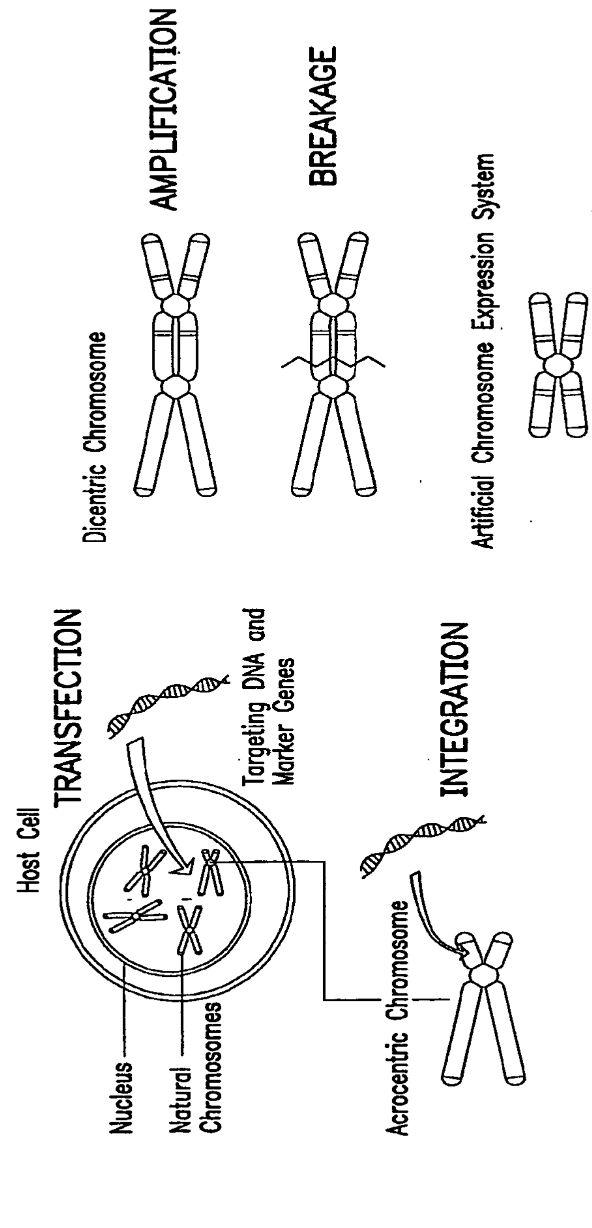


FIG. 1

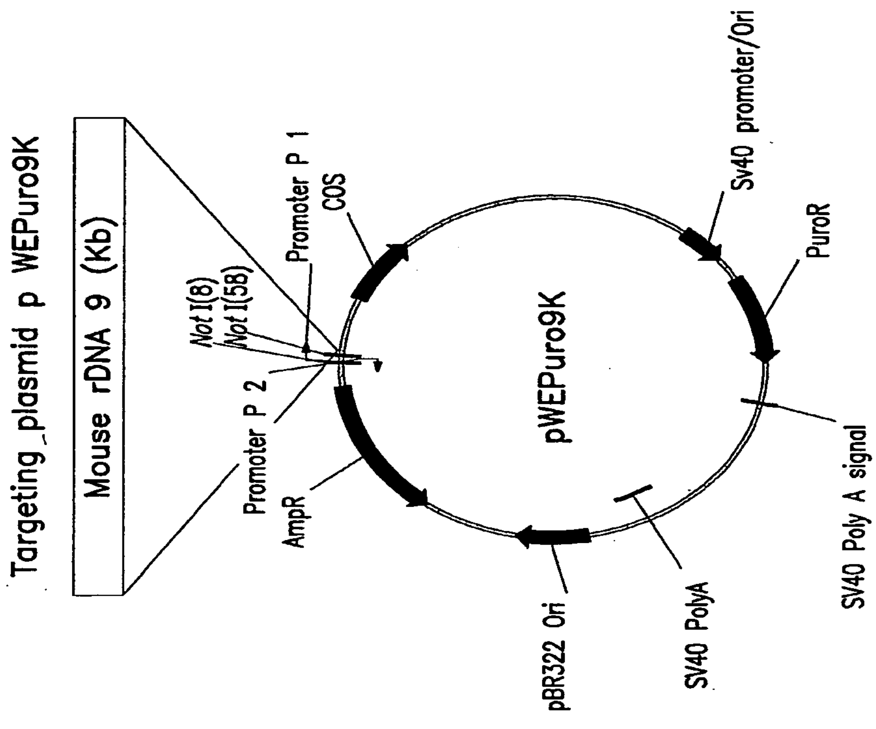

Targeting_plasmid p WEPuro9K
Mouse rDNA 9 (Kb)
Not I(8)
Not I(58)
Promoter P 1
COS
Promoter P 2
AmpR
pWEPuro9K
Sv40 promoter/Ori
PuroR
SV40 Poly A signal
SV40 PolyA
pBR322 Ori

FIG. 2

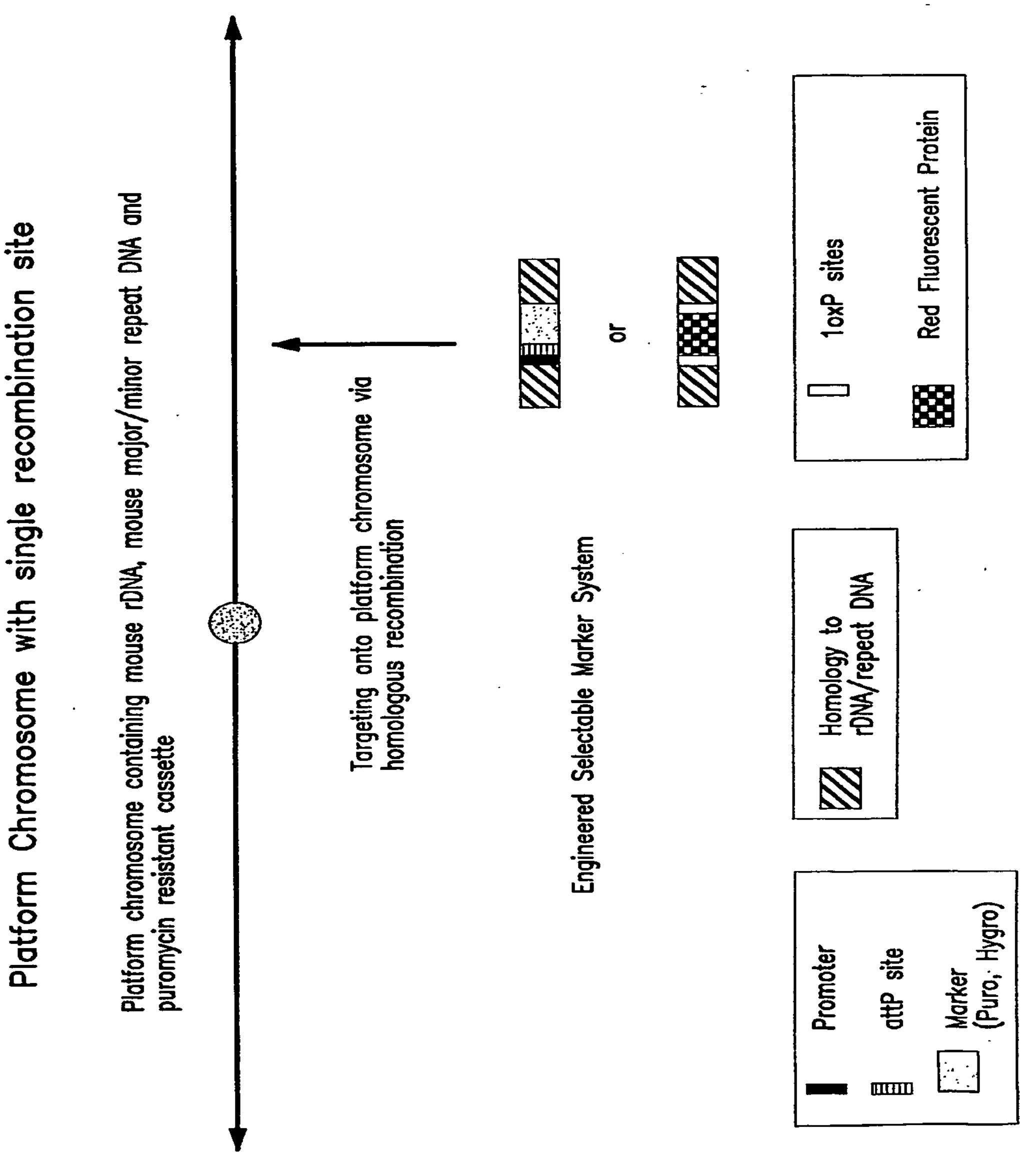
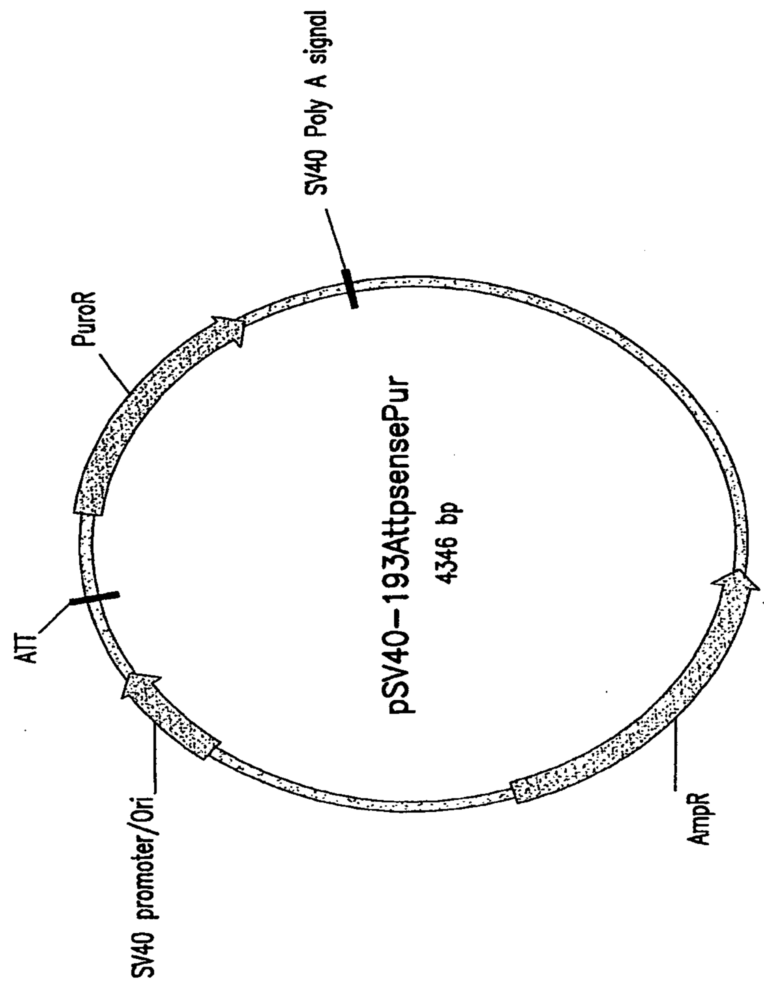

Platform Chromosome with single recombination site
Platform chromosome containing mouse rDNA, mouse major/minor repeat DNA and puromycin resistant cassette
Targeting onto platform chromosome via homologous recombination
Engineered Selectable Marker System
or
Promoter
attP site
Marker (Puro; Hygro)
Homology to rDNA/repeat DNA
1oxP sites
Red Fluorescent Protein

FIG. 3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ATT
SV40 promoter/Ori
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FIG. 4

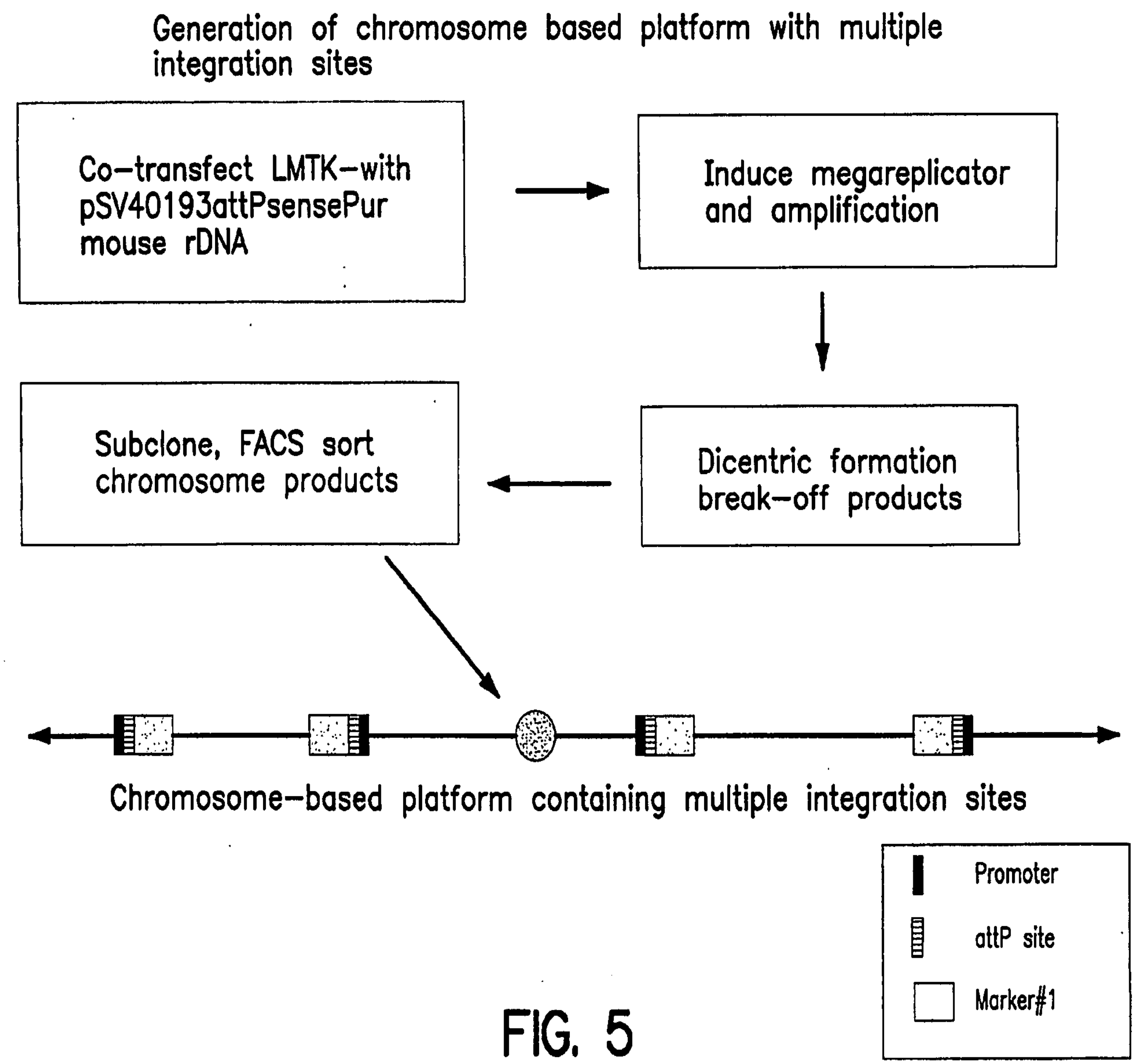

Generation of chromosome based platform with multiple integration sites
Co-transfect LMTK-with pSV40193attPsensePur mouse rDNA
Induce megareplicator and amplification
Dicentric formation break-off products
Subclone, FACS sort chromosome products
Chromosome-based platform containing multiple integration sites
Promoter
attP site
Marker#1

FIG. 5

λ integrase recombination

attP x attB ⇄ attL x attR

	Core Region
attP	CAGCTTTTTTATACTAAGTTG
attB	CTGCTTTTTTATACTAACTTG
attL	CTGCTTTTTTATACTAAGTTG
attR	CAGCTTTTTTATACTAACTTG

FIG. 6

λ INT recombination on artificial chromosome

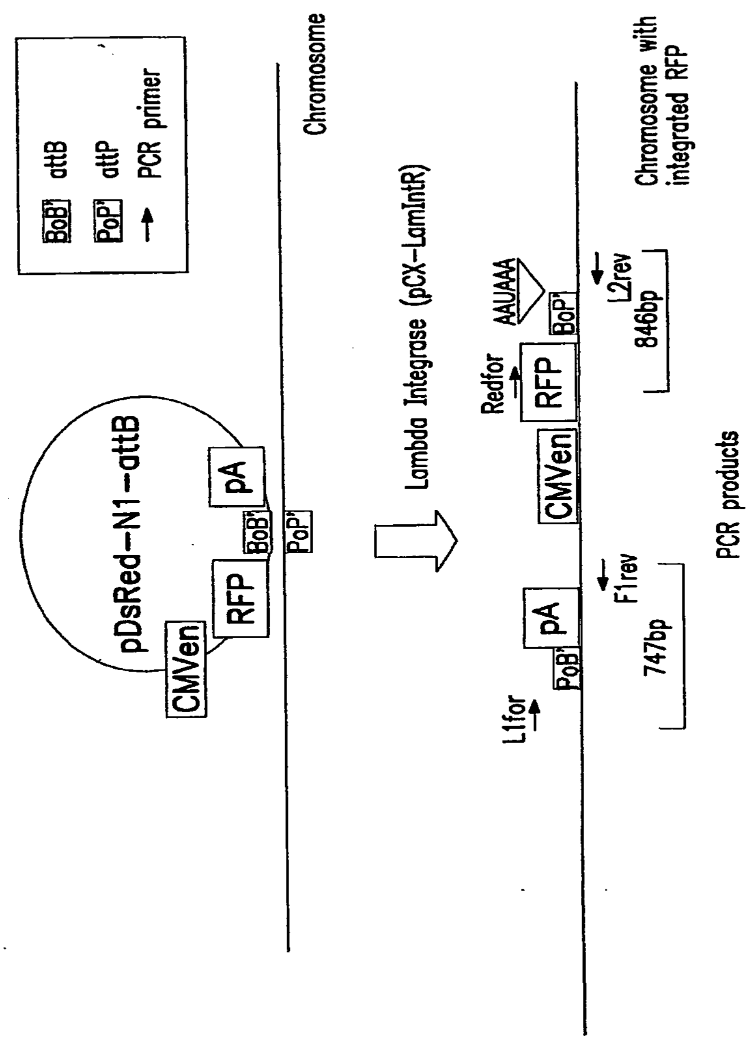


FIG. 7

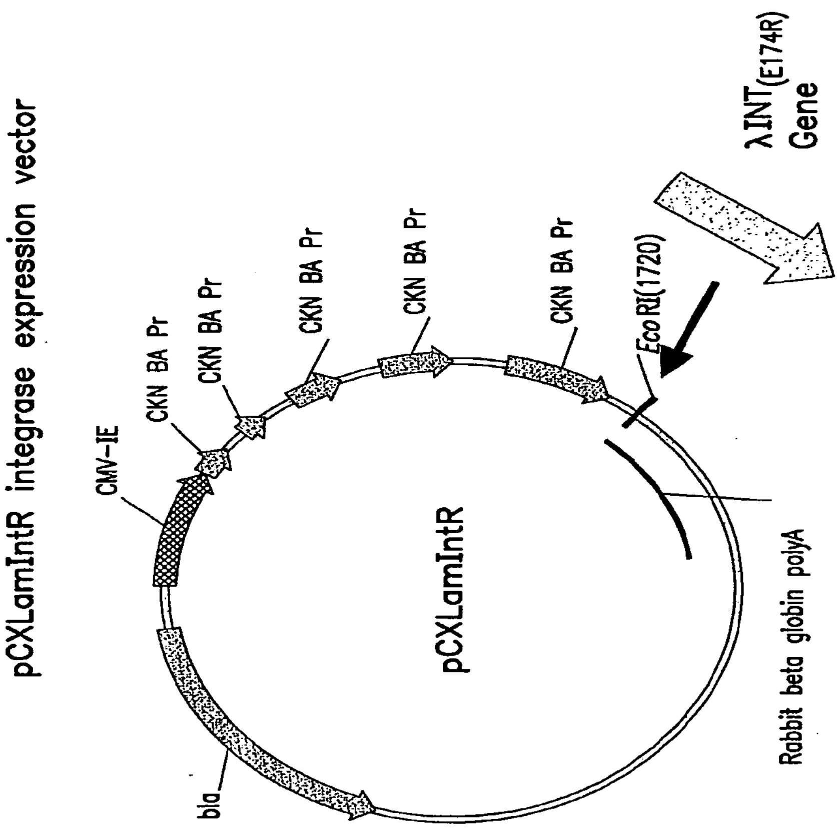

pCXLamIntR integrase expression vector
CMV-IE
CKN BA Pr
CKN BA Pr
CKN BA Pr
CKN BA Pr
CKN BA Pr
EcoRI(1720)
λINT(E174R)
Gene
pCXLamIntR
Rabbit beta globin polyA
bla

FIG. 8

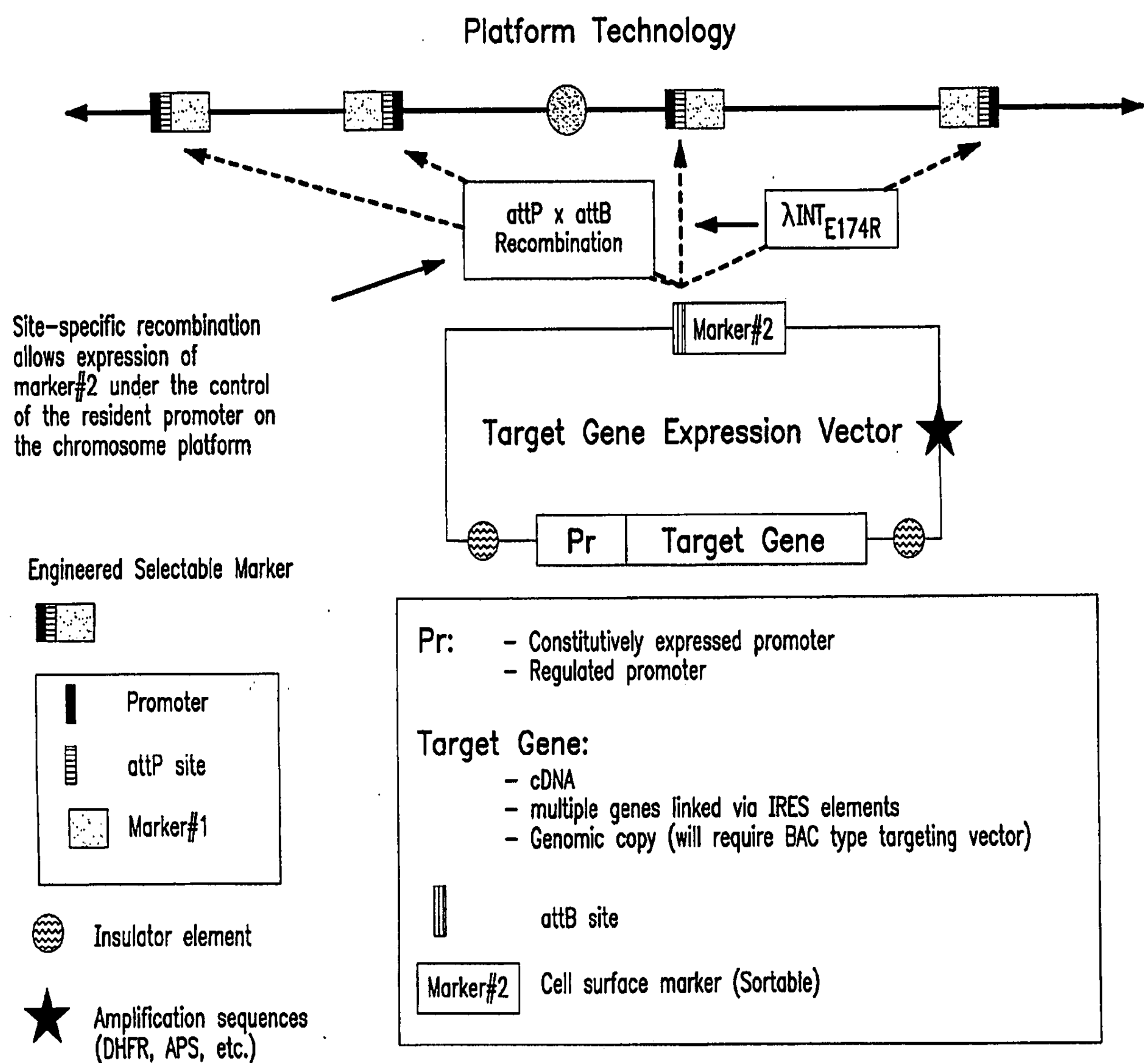
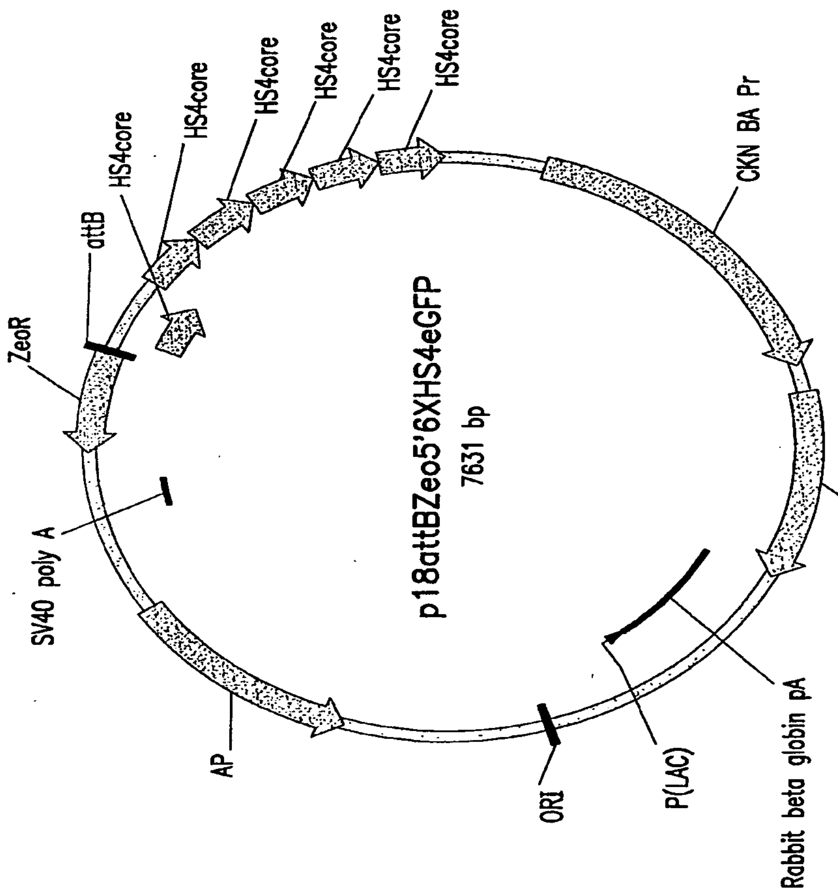

Platform Technology
attP x attB
Recombination
λINT E174R
Site-specific recombination allows expression of marker#2 under the control of the resident promoter on the chromosome platform
Marker#2
Target Gene Expression Vector
Pr
Target Gene
Engineered Selectable Marker
Promoter
attP site
Marker#1
Insulator element
Amplification sequences (DHFR, APS, etc.)
Pr: - Constitutively expressed promoter
- Regulated promoter
Target Gene:
- cDNA
- multiple genes linked via IRES elements
- Genomic copy (will require BAC type targeting vector)
attB site
Marker#2
Cell surface marker (Sortable)

FIG. 9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7631 bp
ZeoR
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HS4core
HS4core
HS4core
HS4core
HS4core
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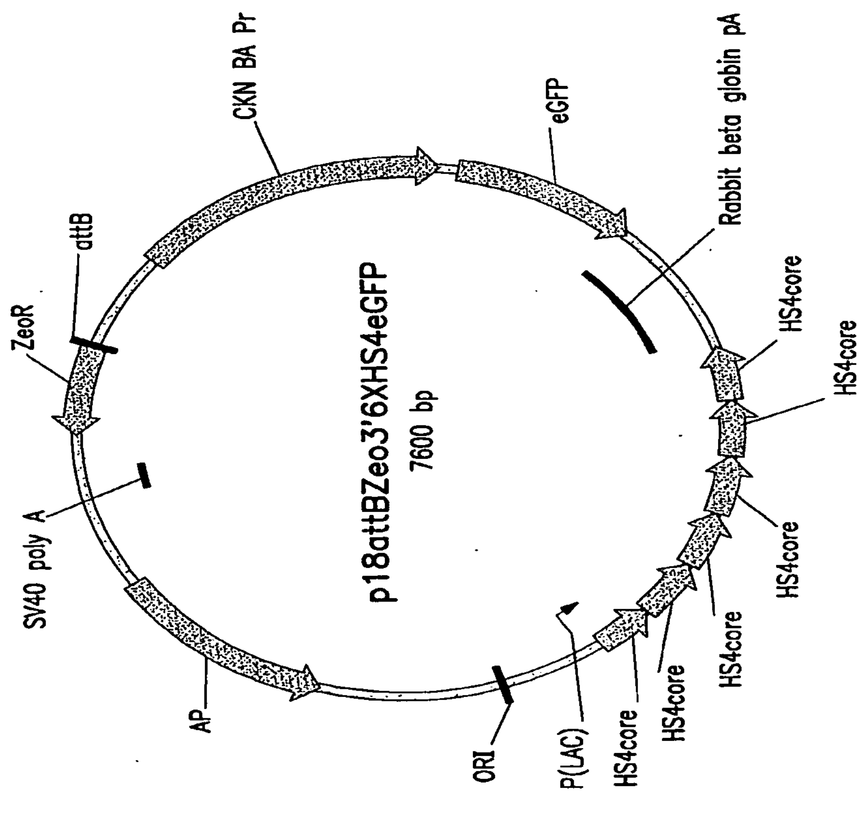
CKN BA Pr
eGFP
Rabbit beta globin pA
P(LAC)
ORI
AP
SV40 poly A

FIG. 10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Rabbit beta globin pA
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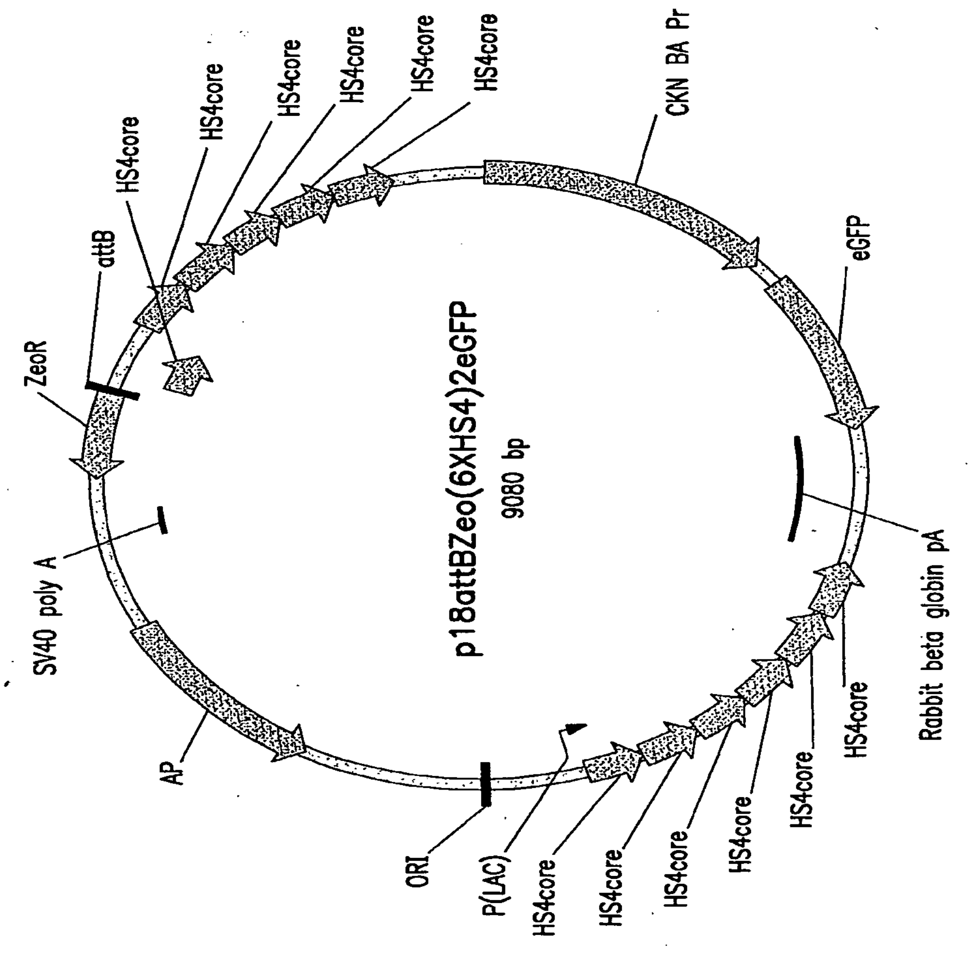
P(LAC)
ORI
AP
SV40 poly A
ZeoR
attB

FIG. 11



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9080 bp
CKN BA Pr
eGFP
Rabbit beta globin pA
HS4core
HS4core
HS4core
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HS4core
HS4core
P(LAC)
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ZeoR
attB
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HS4core
HS4core

FIG. 12

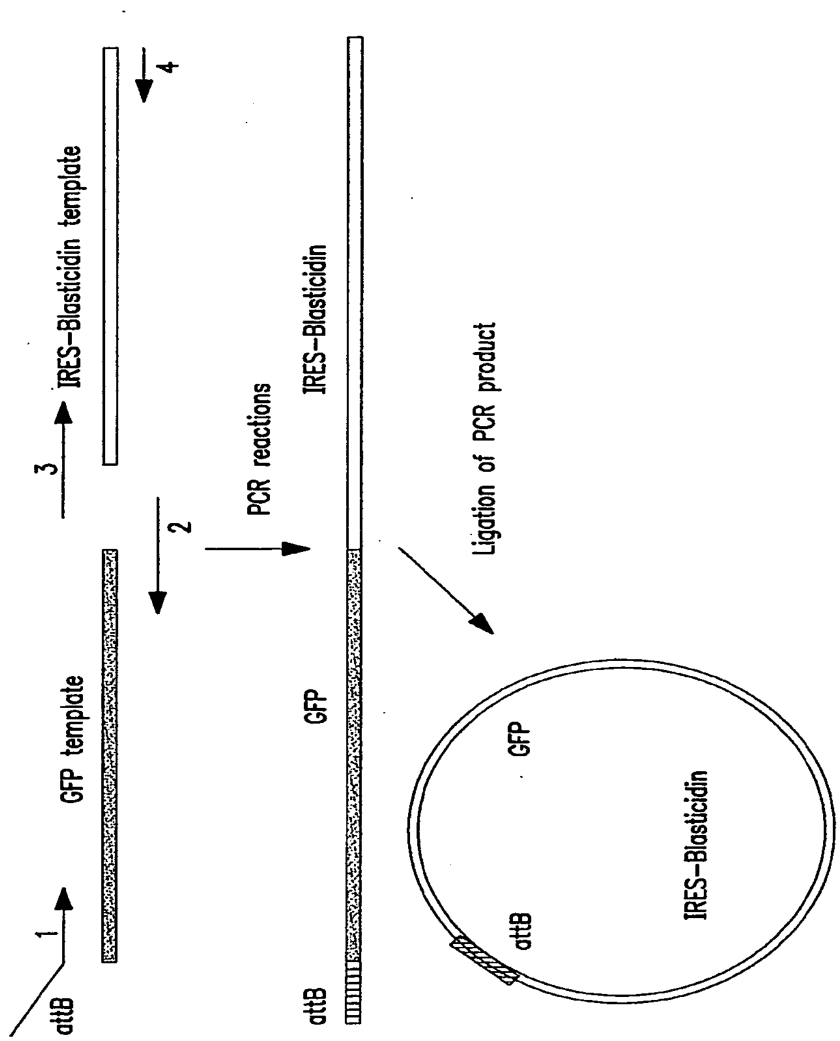

attB
1
GFP template
3
IRES–Blasticidin template
2
4
PCR reactions
attB
GFP
IRES–Blasticidin
Ligation of PCR product
attB
GFP
IRES–Blasticidin

FIG. 13

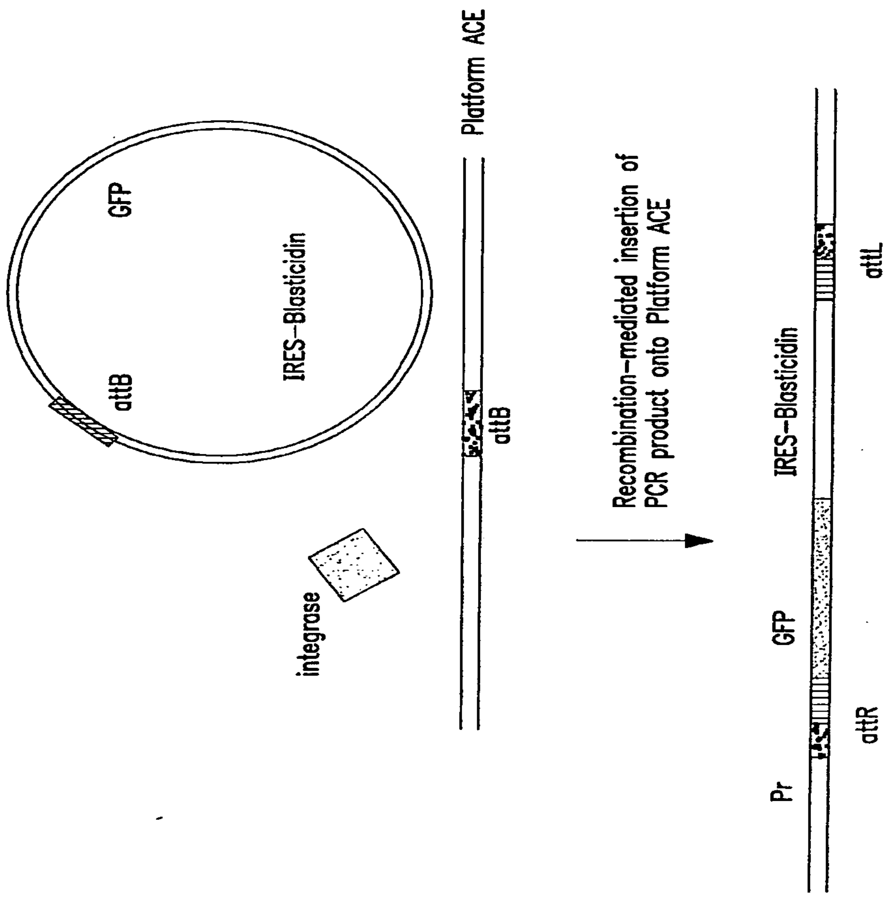
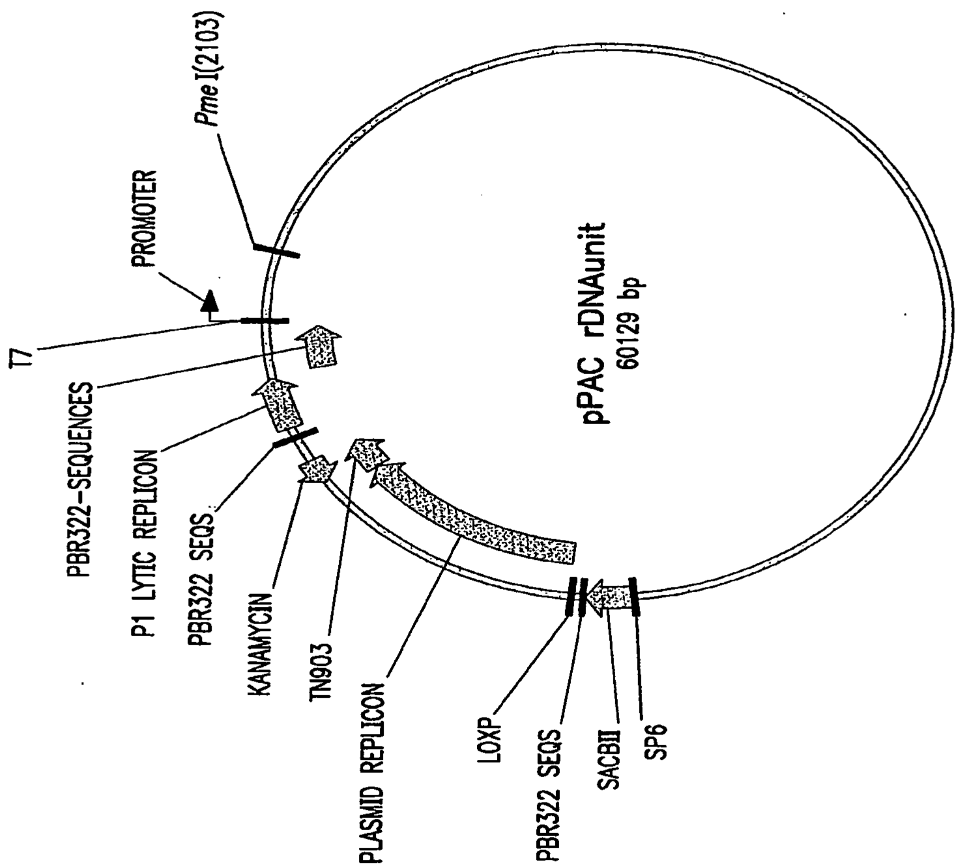

integrase
attB
IRES–Blasticidin
GFP
Platform ACE
attB
Recombination–mediated insertion of PCR product onto Platform ACE
Pr
attR
GFP
IRES–Blasticidin
attL

FIG. 14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60129 bp
Pme I(2103)
PROMOTER
T7
PBR322-SEQUENCES
P1 LYTIC REPLICON
PBR322 SEQS
KANAMYCIN
TN903
PLASMID REPLICON
LOXP
PBR322 SEQS
SACBII
SP6

FIG. 15

SEQUENCE LISTING

<110> CHROMOS MOLECULAR SYSTEMS, INC.
Perkins, Edward
Perez, Carl
Lindenbaum, Michael
Greene, Amy
Leung, Josephine
Fleming, Elena
Stewart, Sandra
Shellard, Joan

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cagacagaca gacagacgtt acaaacaaac acgttgagcc gtgtgccaac acacacacaa 240
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<213> Artificial Sequence

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<213> Artificial Sequence

<220>
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caaggtaacc gtcagtgccg ataagttcaa agttaaacct ggtgttgata ccaacattga 7080
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aattcgcggc cgcaattaac cctcactaaa ggatcc 8136

<210> 32
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<212> DNA
<213> Artificial Sequence

<220>
<223> pNEB193 plasmid

<400> 32
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ttggcgggtg tcggggctgg cttaactatg cggcatcaga gcagattgta ctgagagtgc 180
accatatgcg gtgtgaaata ccgcacagat gcgtaaggag aaaataccgc atcaggcgcc 240
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tacgccagct ggcgaaaggg ggatgtgctg caaggcgatt aagttgggta acgccagggt 360
tttcccagtc acgacgttgt aaaacgacgg ccagtgaatt cgagctcggt acccgggggc 420
gcgccggatc cttaattaag tctagagtcg actgtttaaa cctgcaggca tgcaagcttg 480
gcgtaatcat ggtcatagct gtttcctgtg tgaaattgtt atccgctcac aattccacac 540
aacatacgag ccggaagcat aaagtgtaaa gcctggggtg cctaatgagt gagctaactc 600
acattaattg cgttgcgctc actgcccgct ttccagtcgg gaaacctgtc gtgccagctg 660
cattaatgaa tcggccaacg cgcggggaga ggcggtttgc gtattgggcg ctcttccgct 720
tcctcgctca ctgactcgct gcgctcggtc gttcggctgc ggcgagcggt atcagctcac 780
tcaaaggcgg taatacggtt atccacagaa tcaggggata acgcaggaaa gaacatgtga 840
gcaaaaggcc agcaaaaggc caggaaccgt aaaaaggccg cgttgctggc gtttttccat 900
aggctccgcc cccctgacga gcatcacaaa aatcgacgct caagtcagag gtggcgaaac 960

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cttgagtcca acccggtaag acacgactta tcgccactgg cagcagccac tggtaacagg   1260
attagcagag cgaggtatgt aggcggtgct acagagttct tgaagtggtg gcctaactac   1320
ggctacacta gaaggacagt atttggtatc tgcgctctgc tgaagccagt taccttcgga   1380
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tctacggggt ctgacgctca gtggaacgaa aactcacgtt aagggatttt ggtcatgaga   1560
ttatcaaaaa ggatcttcac ctagatcctt ttaaattaaa aatgaagttt taaatcaatc   1620
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actacgatac gggagggctt accatctggc cccagtgctg caatgatacc gcgagaccca   1800
cgctcaccgg ctccagattt atcagcaata aaccagccag ccggaagggc cgagcgcaga   1860
agtggtcctg caactttatc cgcctccatc cagtctatta attgttgccg ggaagctaga   1920
gtaagtagtt cgccagttaa tagtttgcgc aacgttgttg ccattgctac aggcatcgtg   1980
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cttactgtca tgccatccgt aagatgcttt tctgtgactg gtgagtactc aaccaagtca   2220
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accgcgccac atagcagaac tttaaaagtg ctcatcattg gaaaacgttc ttcggggcga   2340
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aactgatctt cagcatcttt tactttcacc agcgtttctg ggtgagcaaa aacaggaagg   2460
caaaatgccg caaaaaaggg aataagggcg acacggaaat gttgaatact catactcttc   2520
ctttttcaat attattgaag catttatcag ggttattgtc tcatgagcgg atacatattt   2580
gaatgtattt agaaaaataa acaaataggg gttccgcgca catttccccg aaaagtgcca   2640
cctgacgtct aagaaaccat tattatcatg acattaacct ataaaaatag gcgtatcacg   2700
aggccctttc gtc                                                        2713
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<210> 33
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> attP

<400> 33
cagctttttt atactaagtt g 21

<210> 34
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> attB

<400> 34
ctgctttttt atactaactt g 21

<210> 35
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> attL

<400> 35
ctgctttttt atactaagtt g 21

<210> 36
<211> 21
<212> DNA

<213> Artificial Sequence

<220>
<223> attR

<400> 36
cagctttttt atactaactt g 21

<210> 37
<211> 1071
<212> DNA
<213> Artificial Sequence

<220>
<223> Integrase E174R

<221> CDS
<222> (1)...(1071)
<223> Nucleotide sequence encoding Integrase E147R

<400> 37
atg gga aga agg cga agt cat gag cgc cgg gat tta ccc cct aac ctt 48
Met Gly Arg Arg Arg Ser His Glu Arg Arg Asp Leu Pro Pro Asn Leu
1 5 10 15

tat ata aga aac aat gga tat tac tgc tac agg gac cca agg acg ggt 96
Tyr Ile Arg Asn Asn Gly Tyr Tyr Cys Tyr Arg Asp Pro Arg Thr Gly
20 25 30

aaa gag ttt gga tta ggc aga gac agg cga atc gca atc act gaa gct 144
Lys Glu Phe Gly Leu Gly Arg Asp Arg Arg Ile Ala Ile Thr Glu Ala
35 40 45

ata cag gcc aac att gag tta ttt tca gga cac aaa cac aag cct ctg 192
Ile Gln Ala Asn Ile Glu Leu Phe Ser Gly His Lys His Lys Pro Leu
50 55 60

aca gcg aga atc aac agt gat aat tcc gtt acg tta cat tca tgg ctt 240
Thr Ala Arg Ile Asn Ser Asp Asn Ser Val Thr Leu His Ser Trp Leu
65 70 75 80

gat cgc tac gaa aaa atc ctg gcc agc aga gga atc aag cag aag aca 288
Asp Arg Tyr Glu Lys Ile Leu Ala Ser Arg Gly Ile Lys Gln Lys Thr
85 90 95

ctc ata aat tac atg agc aaa att aaa gca ata agg agg ggt ctg cct 336
Leu Ile Asn Tyr Met Ser Lys Ile Lys Ala Ile Arg Arg Gly Leu Pro
100 105 110

gat gct cca ctt gaa gac atc acc aca aaa gaa att gcg gca atg ctc 384
Asp Ala Pro Leu Glu Asp Ile Thr Thr Lys Glu Ile Ala Ala Met Leu
115 120 125

aat gga tac ata gac gag ggc aag gcg gcg tca gcc aag tta atc aga 432
Asn Gly Tyr Ile Asp Glu Gly Lys Ala Ala Ser Ala Lys Leu Ile Arg
130 135 140

tca aca ctg agc gat gca ttc cga gag gca ata gct gaa ggc cat ata 480
Ser Thr Leu Ser Asp Ala Phe Arg Glu Ala Ile Ala Glu Gly His Ile
145 150 155 160

aca aca aac cat gtc gct gcc act cgc gca gca aaa tct aga gta agg 528
Thr Thr Asn His Val Ala Ala Thr Arg Ala Ala Lys Ser Arg Val Arg
165 170 175

aga tca aga ctt acg gct gac gaa tac ctg aaa att tat caa gca gca 576
Arg Ser Arg Leu Thr Ala Asp Glu Tyr Leu Lys Ile Tyr Gln Ala Ala

180 185 190

gaa tca tca cca tgt tgg ctc aga ctt gca atg gaa ctg gct gtt gtt 624
Glu Ser Ser Pro Cys Trp Leu Arg Leu Ala Met Glu Leu Ala Val Val
195 200 205

acc ggg caa cga gtt ggt gat tta tgc gaa atg aag tgg tct gat atc 672
Thr Gly Gln Arg Val Gly Asp Leu Cys Glu Met Lys Trp Ser Asp Ile
210 215 220

gta gat gga tat ctt tat gtc gag caa agc aaa aca ggc gta aaa att 720
Val Asp Gly Tyr Leu Tyr Val Glu Gln Ser Lys Thr Gly Val Lys Ile
225 230 235 240

gcc atc cca aca gca ttg cat att gat gct ctc gga ata tca atg aag 768
Ala Ile Pro Thr Ala Leu His Ile Asp Ala Leu Gly Ile Ser Met Lys
245 250 255

gaa aca ctt gat aaa tgc aaa gag att ctt ggc gga gaa acc ata att 816
Glu Thr Leu Asp Lys Cys Lys Glu Ile Leu Gly Gly Glu Thr Ile Ile
260 265 270

gca tct act cgt cgc gaa ccg ctt tca tcc ggc aca gta tca agg tat 864
Ala Ser Thr Arg Arg Glu Pro Leu Ser Ser Gly Thr Val Ser Arg Tyr
275 280 285

ttt atg cgc gca cga aaa gca tca ggt ctt tcc ttc gaa ggg gat ccg 912
Phe Met Arg Ala Arg Lys Ala Ser Gly Leu Ser Phe Glu Gly Asp Pro
290 295 300

cct acc ttt cac gag ttg cgc agt ttg tct gca aga ctc tat gag aag 960
Pro Thr Phe His Glu Leu Arg Ser Leu Ser Ala Arg Leu Tyr Glu Lys
305 310 315 320

cag ata agc gat aag ttt gct caa cat ctt ctc ggg cat aag tcg gac 1008
Gln Ile Ser Asp Lys Phe Ala Gln His Leu Leu Gly His Lys Ser Asp
325 330 335

acc atg gca tca cag tat cgt gat gac aga ggc agg gag tgg gac aaa 1056
Thr Met Ala Ser Gln Tyr Arg Asp Asp Arg Gly Arg Glu Trp Asp Lys
340 345 350

att gaa atc aaa taa 1071
Ile Glu Ile Lys *
355

<210> 38
<211> 356
<212> PRT
<213> Artificial Sequence

<220>
<223> Integrase E147R

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Tyr Ile Arg Asn Asn Gly Tyr Tyr Cys Tyr Arg Asp Pro Arg Thr Gly
20 25 30
Lys Glu Phe Gly Leu Gly Arg Asp Arg Arg Ile Ala Ile Thr Glu Ala
35 40 45
Ile Gln Ala Asn Ile Glu Leu Phe Ser Gly His Lys His Lys Pro Leu
50 55 60
Thr Ala Arg Ile Asn Ser Asp Asn Ser Val Thr Leu His Ser Trp Leu
65 70 75 80
Asp Arg Tyr Glu Lys Ile Leu Ala Ser Arg Gly Ile Lys Gln Lys Thr

85 90 95
Leu Ile Asn Tyr Met Ser Lys Ile Lys Ala Ile Arg Arg Gly Leu Pro
100 105 110
Asp Ala Pro Leu Glu Asp Ile Thr Thr Lys Glu Ile Ala Ala Met Leu
115 120 125
Asn Gly Tyr Ile Asp Glu Gly Lys Ala Ala Ser Ala Lys Leu Ile Arg
130 135 140
Ser Thr Leu Ser Asp Ala Phe Arg Glu Ala Ile Ala Glu Gly His Ile
145 150 155 160
Thr Thr Asn His Val Ala Ala Thr Arg Ala Ala Lys Ser Arg Val Arg
165 170 175
Arg Ser Arg Leu Thr Ala Asp Glu Tyr Leu Lys Ile Tyr Gln Ala Ala
180 185 190
Glu Ser Ser Pro Cys Trp Leu Arg Leu Ala Met Glu Leu Ala Val Val
195 200 205
Thr Gly Gln Arg Val Gly Asp Leu Cys Glu Met Lys Trp Ser Asp Ile
210 215 220
Val Asp Gly Tyr Leu Tyr Val Glu Gln Ser Lys Thr Gly Val Lys Ile
225 230 235 240
Ala Ile Pro Thr Ala Leu His Ile Asp Ala Leu Gly Ile Ser Met Lys
245 250 255
Glu Thr Leu Asp Lys Cys Lys Glu Ile Leu Gly Gly Glu Thr Ile Ile
260 265 270
Ala Ser Thr Arg Arg Glu Pro Leu Ser Ser Gly Thr Val Ser Arg Tyr
275 280 285
Phe Met Arg Ala Arg Lys Ala Ser Gly Leu Ser Phe Glu Gly Asp Pro
290 295 300
Pro Thr Phe His Glu Leu Arg Ser Leu Ser Ala Arg Leu Tyr Glu Lys
305 310 315 320
Gln Ile Ser Asp Lys Phe Ala Gln His Leu Leu Gly His Lys Ser Asp
325 330 335
Thr Met Ala Ser Gln Tyr Arg Asp Asp Arg Gly Arg Glu Trp Asp Lys
340 345 350
Ile Glu Ile Lys
355

<210> 39
<211> 876
<212> DNA
<213> Discosoma species

<220>
<221> CDS
<222> (45)...(737)
<223> Nucleotide sequence encoding red flourescent
protein (FP593)

<300>
<308> GenBank AF272711
<309> 2000-09-26

<400> 39
agtttcagcc agtgacaggg tgagctgcca ggtattctaa caag atg agt tgt tcc 56
Met Ser Cys Ser
1

aag aat gtg atc aag gag ttc atg agg ttc aag gtt cgt atg gaa gga 104
Lys Asn Val Ile Lys Glu Phe Met Arg Phe Lys Val Arg Met Glu Gly
5 10 15 20

acg gtc aat ggg cac gag ttt gaa ata aaa ggc gaa ggt gaa ggg agg 152
Thr Val Asn Gly His Glu Phe Glu Ile Lys Gly Glu Gly Glu Gly Arg
25 30 35

cct tac gaa ggt cac tgt tcc gta aag ctt atg gta acc aag ggt gga 200
Pro Tyr Glu Gly His Cys Ser Val Lys Leu Met Val Thr Lys Gly Gly

40 45 50

cct ttg cca ttt gct ttt gat att ttg tca cca caa ttt cag tat gga 248
Pro Leu Pro Phe Ala Phe Asp Ile Leu Ser Pro Gln Phe Gln Tyr Gly
55 60 65

agc aag gta tat gtc aaa cac cct gcc gac ata cca gac tat aaa aag 296
Ser Lys Val Tyr Val Lys His Pro Ala Asp Ile Pro Asp Tyr Lys Lys
70 75 80

ctg tca ttt cct gag gga ttt aaa tgg gaa agg gtc atg aac ttt gaa 344
Leu Ser Phe Pro Glu Gly Phe Lys Trp Glu Arg Val Met Asn Phe Glu
85 90 95 100

gac ggt ggc gtg gtt act gta tcc caa gat tcc agt ttg aaa gac ggc 392
Asp Gly Gly Val Val Thr Val Ser Gln Asp Ser Ser Leu Lys Asp Gly
105 110 115

tgt ttc atc tac gag gtc aag ttc att ggg gtg aac ttt cct tct gat 440
Cys Phe Ile Tyr Glu Val Lys Phe Ile Gly Val Asn Phe Pro Ser Asp
120 125 130

gga cct gtt atg cag agg agg aca cgg ggc tgg gaa gcc agc tct gag 488
Gly Pro Val Met Gln Arg Arg Thr Arg Gly Trp Glu Ala Ser Ser Glu
135 140 145

cgt ttg tat cct cgt gat ggg gtg ctg aaa gga gac atc cat atg gct 536
Arg Leu Tyr Pro Arg Asp Gly Val Leu Lys Gly Asp Ile His Met Ala
150 155 160

ctg agg ctg gaa gga ggc ggc cat tac ctc gtt gaa ttc aaa agt att 584
Leu Arg Leu Glu Gly Gly Gly His Tyr Leu Val Glu Phe Lys Ser Ile
165 170 175 180

tac atg gta aag aag cct tca gtg cag ttg cca ggc tac tat tat gtt 632
Tyr Met Val Lys Lys Pro Ser Val Gln Leu Pro Gly Tyr Tyr Tyr Val
185 190 195

gac tcc aaa ctg gat atg acg agc cac aac gaa gat tac aca gtc gtt 680
Asp Ser Lys Leu Asp Met Thr Ser His Asn Glu Asp Tyr Thr Val Val
200 205 210

gag cag tat gaa aaa acc cag gga cgc cac cat ccg ttc att aag cct 728
Glu Gln Tyr Glu Lys Thr Gln Gly Arg His His Pro Phe Ile Lys Pro
215 220 225

ctg cag tga actcggctca gtcatggatt agcggtaatg gccacaaaag 777
Leu Gln *
230

gcacgatgat cgtttttag gaatgcagcc aaaaattgaa ggttatgaca gtagaaatac 837
aagcaacagg ctttgcttat taaacatgta attgaaaac 876

<210> 40
<211> 230
<212> PRT
<213> Discosoma species

<400> 40
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1 5 10 15
Arg Met Glu Gly Thr Val Asn Gly His Glu Phe Glu Ile Lys Gly Glu
20 25 30
Gly Glu Gly Arg Pro Tyr Glu Gly His Cys Ser Val Lys Leu Met Val
35 40 45
Thr Lys Gly Gly Pro Leu Pro Phe Ala Phe Asp Ile Leu Ser Pro Gln
50 55 60

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Phe Gln Tyr Gly Ser Lys Val Tyr Val Lys His Pro Ala Asp Ile Pro
65                  70                  75                  80
Asp Tyr Lys Lys Leu Ser Phe Pro Glu Gly Phe Lys Trp Glu Arg Val
                85                  90                  95
Met Asn Phe Glu Asp Gly Gly Val Val Thr Val Ser Gln Asp Ser Ser
            100                 105                 110
Leu Lys Asp Gly Cys Phe Ile Tyr Glu Val Lys Phe Ile Gly Val Asn
        115                 120                 125
Phe Pro Ser Asp Gly Pro Val Met Gln Arg Arg Thr Arg Gly Trp Glu
    130                 135                 140
Ala Ser Ser Glu Arg Leu Tyr Pro Arg Asp Gly Val Leu Lys Gly Asp
145                 150                 155                 160
Ile His Met Ala Leu Arg Leu Glu Gly Gly Gly His Tyr Leu Val Glu
                165                 170                 175
Phe Lys Ser Ile Tyr Met Val Lys Lys Pro Ser Val Gln Leu Pro Gly
            180                 185                 190
Tyr Tyr Tyr Val Asp Ser Lys Leu Asp Met Thr Ser His Asn Glu Asp
        195                 200                 205
Tyr Thr Val Val Glu Gln Tyr Glu Lys Thr Gln Gly Arg His His Pro
    210                 215                 220
Phe Ile Lys Pro Leu Gln
225                 230
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<210> 41
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> m-att;

<221> misc_difference
<222> 18
<223> n is a or g or c or t/u

<400> 41
rkycwgcttt yktrtacnaa stsgb 25

<210> 42
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> m-attB;

<221> misc_difference
<222> 18
<223> n is a or g or c or t/u

<400> 42
agccwgcttt yktrtacnaa ctsgb 25

<210> 43
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> m-attR

<221> misc_difference
<222> 18
<223> n is a or g or c or t/u

<400> 43
gttcagcttt cktrtacnaa ctsgb 25

<210> 44
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> m-attL

<221> misc_difference
<222> 18
<223> n is a or g or c or t/u

<400> 44
agccwgcttt cktrtacnaa gtsgb 25

<210> 45
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> m-attP1

<221> misc_difference
<222> 18
<223> n is a or g or c or t/u

<400> 45
gttcagcttt yktrtacnaa gtsgb 25

<210> 46
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attB1

<400> 46
agcctgcttt tttgtacaaa cttgt 25

<210> 47
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attB2

<400> 47
agcctgcttt cttgtacaaa cttgt 25

<210> 48
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attB3

<400> 48
acccagcttt cttgtacaaa cttgt 25

<210> 49

<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attR1

<400> 49
gttcagcttt tttgtacaaa cttgt 25

<210> 50
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attR2

<400> 50
gttcagcttt cttgtacaaa cttgt 25

<210> 51
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attR3

<400> 51
gttcagcttt cttgtacaaa gttgg 25

<210> 52
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attL1

<400> 52
agcctgcttt tttgtacaaa gttgg 25

<210> 53
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attL2

<400> 53
agcctgcttt cttgtacaaa gttgg 25

<210> 54
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attL3

<400> 54
acccagcttt cttgtacaaa gttgg 25

<210> 55
<211> 25

<212> DNA
<213> Artificial Sequence

<220>
<223> attP1

<400> 55
gttcagcttt tttgtacaaa gttgg 25

<210> 56
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> attP2,P3

<400> 56
gttcagcttt cttgtacaaa gttgg 25

<210> 57
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Lox P site

<400> 57
ataacttcgt ataatgtatg ctatacgaag ttat 34

<210> 58
<211> 1032
<212> DNA
<213> Escherichia coli

<220>
<221> CDS
<222> (1)...(1032)
<223> nucleotide sequence encoding Cre recombinase

<400> 58
atg tcc aat tta ctg acc gta cac caa aat ttg cct gca tta ccg gtc 48
Met Ser Asn Leu Leu Thr Val His Gln Asn Leu Pro Ala Leu Pro Val
1 5 10 15

gat gca acg agt gat gag gtt cgc aag aac ctg atg gac atg ttc agg 96
Asp Ala Thr Ser Asp Glu Val Arg Lys Asn Leu Met Asp Met Phe Arg
20 25 30

gat cgc cag gcg ttt tct gag cat acc tgg aaa atg ctt ctg tcc gtt 144
Asp Arg Gln Ala Phe Ser Glu His Thr Trp Lys Met Leu Leu Ser Val
35 40 45

tgc cgg tcg tgg gcg gca tgg tgc aag ttg aat aac cgg aaa tgg ttt 192
Cys Arg Ser Trp Ala Ala Trp Cys Lys Leu Asn Asn Arg Lys Trp Phe
50 55 60

ccc gca gaa cct gaa gat gtt cgc gat tat ctt cta tat ctt cag gcg 240
Pro Ala Glu Pro Glu Asp Val Arg Asp Tyr Leu Leu Tyr Leu Gln Ala
65 70 75 80

cgc ggt ctg gca gta aaa act atc cag caa cat ttg ggc cag cta aac 288
Arg Gly Leu Ala Val Lys Thr Ile Gln Gln His Leu Gly Gln Leu Asn
85 90 95

atg ctt cat cgt cgg tcc ggg ctg cca cga cca agt gac agc aat gct 336

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Met Leu His Arg Arg Ser Gly Leu Pro Arg Pro Ser Asp Ser Asn Ala
            100                 105                 110

gtt tca ctg gtt atg cgg cgg atc cga aaa gaa aac gtt gat gcc ggt      384
Val Ser Leu Val Met Arg Arg Ile Arg Lys Glu Asn Val Asp Ala Gly
        115                 120                 125

gaa cgt gca aaa cag gct cta gcg ttc gaa cgc act gat ttc gac cag      432
Glu Arg Ala Lys Gln Ala Leu Ala Phe Glu Arg Thr Asp Phe Asp Gln
    130                 135                 140

gtt cgt tca ctc atg gaa aat agc gat cgc tgc cag gat ata cgt aat      480
Val Arg Ser Leu Met Glu Asn Ser Asp Arg Cys Gln Asp Ile Arg Asn
145                 150                 155                 160

ctg gca ttt ctg ggg att gct tat aac acc ctg tta cgt ata gcc gaa      528
Leu Ala Phe Leu Gly Ile Ala Tyr Asn Thr Leu Leu Arg Ile Ala Glu
                165                 170                 175

att gcc agg atc agg gtt aaa gat atc tca cgt act gac ggt ggg aga      576
Ile Ala Arg Ile Arg Val Lys Asp Ile Ser Arg Thr Asp Gly Gly Arg
            180                 185                 190

atg tta atc cat att ggc aga acg aaa acg ctg gtt agc acc gca ggt      624
Met Leu Ile His Ile Gly Arg Thr Lys Thr Leu Val Ser Thr Ala Gly
        195                 200                 205

gta gag aag gca ctt agc ctg ggg gta act aaa ctg gtc gag cga tgg      672
Val Glu Lys Ala Leu Ser Leu Gly Val Thr Lys Leu Val Glu Arg Trp
    210                 215                 220

att tcc gtc tct ggt gta gct gat gat ccg aat aac tac ctg ttt tgc      720
Ile Ser Val Ser Gly Val Ala Asp Asp Pro Asn Asn Tyr Leu Phe Cys
225                 230                 235                 240

cgg gtc aga aaa aat ggt gtt gcc gcg cca tct gcc acc agc cag cta      768
Arg Val Arg Lys Asn Gly Val Ala Ala Pro Ser Ala Thr Ser Gln Leu
                245                 250                 255

tca act cgc gcc ctg gaa ggg att ttt gaa gca act cat cga ttg att      816
Ser Thr Arg Ala Leu Glu Gly Ile Phe Glu Ala Thr His Arg Leu Ile
            260                 265                 270

tac ggc gct aag gat gac tct ggt cag aga tac ctg gcc tgg tct gga      864
Tyr Gly Ala Lys Asp Asp Ser Gly Gln Arg Tyr Leu Ala Trp Ser Gly
        275                 280                 285

cac agt gcc cgt gtc gga gcc gcg cga gat atg gcc cgc gct gga gtt      912
His Ser Ala Arg Val Gly Ala Ala Arg Asp Met Ala Arg Ala Gly Val
    290                 295                 300

tca ata ccg gag atc atg caa gct ggt ggc tgg acc aat gta aat att      960
Ser Ile Pro Glu Ile Met Gln Ala Gly Gly Trp Thr Asn Val Asn Ile
305                 310                 315                 320

gtc atg aac tat atc cgt aac ctg gat agt gaa aca ggg gca atg gtg     1008
Val Met Asn Tyr Ile Arg Asn Leu Asp Ser Glu Thr Gly Ala Met Val
                325                 330                 335

cgc ctg ctg gaa gat ggc gat tag                                     1032
Arg Leu Leu Glu Asp Gly Asp  *
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<210> 59
<211> 343
<212> PRT

<213> Escherichia coli

<400> 59
Met Ser Asn Leu Leu Thr Val His Gln Asn Leu Pro Ala Leu Pro Val
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Asp Ala Thr Ser Asp Glu Val Arg Lys Asn Leu Met Asp Met Phe Arg
20 25 30
Asp Arg Gln Ala Phe Ser Glu His Thr Trp Lys Met Leu Leu Ser Val
35 40 45
Cys Arg Ser Trp Ala Ala Trp Cys Lys Leu Asn Asn Arg Lys Trp Phe
50 55 60
Pro Ala Glu Pro Glu Asp Val Arg Asp Tyr Leu Leu Tyr Leu Gln Ala
65 70 75 80
Arg Gly Leu Ala Val Lys Thr Ile Gln Gln His Leu Gly Gln Leu Asn
85 90 95
Met Leu His Arg Arg Ser Gly Leu Pro Arg Pro Ser Asp Ser Asn Ala
100 105 110
Val Ser Leu Val Met Arg Arg Ile Arg Lys Glu Asn Val Asp Ala Gly
115 120 125
Glu Arg Ala Lys Gln Ala Leu Ala Phe Glu Arg Thr Asp Phe Asp Gln
130 135 140
Val Arg Ser Leu Met Glu Asn Ser Asp Arg Cys Gln Asp Ile Arg Asn
145 150 155 160
Leu Ala Phe Leu Gly Ile Ala Tyr Asn Thr Leu Leu Arg Ile Ala Glu
165 170 175
Ile Ala Arg Ile Arg Val Lys Asp Ile Ser Arg Thr Asp Gly Gly Arg
180 185 190
Met Leu Ile His Ile Gly Arg Thr Lys Thr Leu Val Ser Thr Ala Gly
195 200 205
Val Glu Lys Ala Leu Ser Leu Gly Val Thr Lys Leu Val Glu Arg Trp
210 215 220
Ile Ser Val Ser Gly Val Ala Asp Asp Pro Asn Asn Tyr Leu Phe Cys
225 230 235 240
Arg Val Arg Lys Asn Gly Val Ala Ala Pro Ser Ala Thr Ser Gln Leu
245 250 255
Ser Thr Arg Ala Leu Glu Gly Ile Phe Glu Ala Thr His Arg Leu Ile
260 265 270
Tyr Gly Ala Lys Asp Asp Ser Gly Gln Arg Tyr Leu Ala Trp Ser Gly
275 280 285
His Ser Ala Arg Val Gly Ala Ala Arg Asp Met Ala Arg Ala Gly Val
290 295 300
Ser Ile Pro Glu Ile Met Gln Ala Gly Gly Trp Thr Asn Val Asn Ile
305 310 315 320
Val Met Asn Tyr Ile Arg Asn Leu Asp Ser Glu Thr Gly Ala Met Val
325 330 335
Arg Leu Leu Glu Asp Gly Asp
340

<210> 60
<211> 1272
<212> DNA
<213> Saccharomyces cerevisiae

<220>
<221> CDS
<222> (1)...(1272)
<223> nucleotide sequence encoding Flip recombinase

<400> 60
atg cca caa ttt ggt ata tta tgt aaa aca cca cct aag gtg ctt gtt 48
Met Pro Gln Phe Gly Ile Leu Cys Lys Thr Pro Pro Lys Val Leu Val
1 5 10 15

cgt cag ttt gtg gaa agg ttt gaa aga cct tca ggt gag aaa ata gca 96
Arg Gln Phe Val Glu Arg Phe Glu Arg Pro Ser Gly Glu Lys Ile Ala
20 25 30

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tta tgt gct gct gaa cta acc tat tta tgt tgg atg att aca cat aac      144
Leu Cys Ala Ala Glu Leu Thr Tyr Leu Cys Trp Met Ile Thr His Asn
        35                  40                  45

gga aca gca atc aag aga gcc aca ttc atg agc tat aat act atc ata      192
Gly Thr Ala Ile Lys Arg Ala Thr Phe Met Ser Tyr Asn Thr Ile Ile
    50                  55                  60

agc aat tcg ctg agt ttc gat att gtc aat aaa tca ctc cag ttt aaa      240
Ser Asn Ser Leu Ser Phe Asp Ile Val Asn Lys Ser Leu Gln Phe Lys
65                  70                  75                  80

tac aag acg caa aaa gca aca att ctg gaa gcc tca tta aag aaa ttg      288
Tyr Lys Thr Gln Lys Ala Thr Ile Leu Glu Ala Ser Leu Lys Lys Leu
                85                  90                  95

att cct gct tgg gaa ttt aca att att cct tac tat gga caa aaa cat      336
Ile Pro Ala Trp Glu Phe Thr Ile Ile Pro Tyr Tyr Gly Gln Lys His
            100                 105                 110

caa tct gat atc act gat att gta agt agt ttg caa tta cag ttc gaa      384
Gln Ser Asp Ile Thr Asp Ile Val Ser Ser Leu Gln Leu Gln Phe Glu
        115                 120                 125

tca tcg gaa gaa gca gat aag gga aat agc cac agt aaa aaa atg ctt      432
Ser Ser Glu Glu Ala Asp Lys Gly Asn Ser His Ser Lys Lys Met Leu
    130                 135                 140

aaa gca ctt cta agt gag ggt gaa agc atc tgg gag atc act gag aaa      480
Lys Ala Leu Leu Ser Glu Gly Glu Ser Ile Trp Glu Ile Thr Glu Lys
145                 150                 155                 160

ata cta aat tcg ttt gag tat act tcg aga ttt aca aaa aca aaa act      528
Ile Leu Asn Ser Phe Glu Tyr Thr Ser Arg Phe Thr Lys Thr Lys Thr
                165                 170                 175

tta tac caa ttc ctc ttc cta gct act ttc atc aat tgt gga aga ttc      576
Leu Tyr Gln Phe Leu Phe Leu Ala Thr Phe Ile Asn Cys Gly Arg Phe
            180                 185                 190

agc gat att aag aac gtt gat ccg aaa tca ttt aaa tta gtc caa aat      624
Ser Asp Ile Lys Asn Val Asp Pro Lys Ser Phe Lys Leu Val Gln Asn
        195                 200                 205

aag tat ctg gga gta ata atc cag tgt tta gtg aca gag aca aag aca      672
Lys Tyr Leu Gly Val Ile Ile Gln Cys Leu Val Thr Glu Thr Lys Thr
    210                 215                 220

agc gtt agt agg cac ata tac ttc ttt agc gca agg ggt agg atc gat      720
Ser Val Ser Arg His Ile Tyr Phe Phe Ser Ala Arg Gly Arg Ile Asp
225                 230                 235                 240

cca ctt gta tat ttg gat gaa ttt ttg agg aat tct gaa cca gtc cta      768
Pro Leu Val Tyr Leu Asp Glu Phe Leu Arg Asn Ser Glu Pro Val Leu
                245                 250                 255

aaa cga gta aat agg acc ggc aat tct tca agc aat aaa cag gaa tac      816
Lys Arg Val Asn Arg Thr Gly Asn Ser Ser Ser Asn Lys Gln Glu Tyr
            260                 265                 270

caa tta tta aaa gat aac tta gtc aga tcg tac aat aaa gct ttg aag      864
Gln Leu Leu Lys Asp Asn Leu Val Arg Ser Tyr Asn Lys Ala Leu Lys
        275                 280                 285

aaa aat gcg cct tat tca atc ttt gct ata aaa aat ggc cca aaa tct      912
Lys Asn Ala Pro Tyr Ser Ile Phe Ala Ile Lys Asn Gly Pro Lys Ser
    290                 295                 300
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cac att gga aga cat ttg atg acc tca ttt ctt tca atg aag ggc cta 960
His Ile Gly Arg His Leu Met Thr Ser Phe Leu Ser Met Lys Gly Leu
305 310 315 320

acg gag ttg act aat gtt gtg gga aat tgg agc gat aag cgt gct tct 1008
Thr Glu Leu Thr Asn Val Val Gly Asn Trp Ser Asp Lys Arg Ala Ser
325 330 335

gcc gtg gcc agg aca acg tat act cat cag ata aca gca ata cct gat 1056
Ala Val Ala Arg Thr Thr Tyr Thr His Gln Ile Thr Ala Ile Pro Asp
340 345 350

cac tac ttc gca cta gtt tct cgg tac tat gca tat gat cca ata tca 1104
His Tyr Phe Ala Leu Val Ser Arg Tyr Tyr Ala Tyr Asp Pro Ile Ser
355 360 365

aag gaa atg ata gca ttg aag gat gag act aat cca att gag gag tgg 1152
Lys Glu Met Ile Ala Leu Lys Asp Glu Thr Asn Pro Ile Glu Glu Trp
370 375 380

cag cat ata gaa cag cta aag ggt agt gct gaa gga agc ata cga tac 1200
Gln His Ile Glu Gln Leu Lys Gly Ser Ala Glu Gly Ser Ile Arg Tyr
385 390 395 400

ccc gca tgg aat ggg ata ata tca cag gag gta cta gac tac ctt tca 1248
Pro Ala Trp Asn Gly Ile Ile Ser Gln Glu Val Leu Asp Tyr Leu Ser
405 410 415

tcc tac ata aat aga cgc ata taa 1272
Ser Tyr Ile Asn Arg Arg Ile *
420

<210> 61
<211> 422
<212> PRT
<213> Saccharomyces cerevisiae

<400> 61
Pro Gln Phe Gly Ile Leu Cys Lys Thr Pro Pro Lys Val Leu Val Arg
1 5 10 15
Gln Phe Val Glu Arg Phe Glu Arg Pro Ser Gly Glu Lys Ile Ala Leu
20 25 30
Cys Ala Ala Glu Leu Thr Tyr Leu Cys Trp Met Ile Thr His Asn Gly
35 40 45
Thr Ala Ile Lys Arg Ala Thr Phe Met Ser Tyr Asn Thr Ile Ile Ser
50 55 60
Asn Ser Leu Ser Phe Asp Ile Val Asn Lys Ser Leu Gln Phe Lys Tyr
65 70 75 80
Lys Thr Gln Lys Ala Thr Ile Leu Glu Ala Ser Leu Lys Lys Leu Ile
85 90 95
Pro Ala Trp Glu Phe Thr Ile Ile Pro Tyr Tyr Gly Gln Lys His Gln
100 105 110
Ser Asp Ile Thr Asp Ile Val Ser Ser Leu Gln Leu Gln Phe Glu Ser
115 120 125
Ser Glu Glu Ala Asp Lys Gly Asn Ser His Ser Lys Lys Met Leu Lys
130 135 140
Ala Leu Leu Ser Glu Gly Glu Ser Ile Trp Glu Ile Thr Glu Lys Ile
145 150 155 160
Leu Asn Ser Phe Glu Tyr Thr Ser Arg Phe Thr Lys Thr Lys Thr Leu
165 170 175
Tyr Gln Phe Leu Phe Leu Ala Thr Phe Ile Asn Cys Gly Arg Phe Ser
180 185 190
Asp Ile Lys Asn Val Asp Pro Lys Ser Phe Lys Leu Val Gln Asn Lys
195 200 205
Tyr Leu Gly Val Ile Ile Gln Cys Leu Val Thr Glu Thr Lys Thr Ser

210 215 220
Val Ser Arg His Ile Tyr Phe Phe Ser Ala Arg Gly Arg Ile Asp Pro
225 230 235 240
Leu Val Tyr Leu Asp Glu Phe Leu Arg Asn Ser Glu Pro Val Leu Lys
245 250 255
Arg Val Asn Arg Thr Gly Asn Ser Ser Ser Asn Lys Gln Glu Tyr Gln
260 265 270
Leu Leu Lys Asp Asn Leu Val Arg Ser Tyr Asn Lys Ala Leu Lys Lys
275 280 285
Asn Ala Pro Tyr Ser Ile Phe Ala Ile Lys Asn Gly Pro Lys Ser His
290 295 300
Ile Gly Arg His Leu Met Thr Ser Phe Leu Ser Met Lys Gly Leu Thr
305 310 315 320
Glu Leu Thr Asn Val Val Gly Asn Trp Ser Asp Lys Arg Ala Ser Ala
325 330 335
Val Ala Arg Thr Thr Tyr Thr His Gln Ile Thr Ala Ile Pro Asp His
340 345 350
Tyr Phe Ala Leu Val Ser Arg Tyr Tyr Ala Tyr Asp Pro Ile Ser Lys
355 360 365
Glu Met Ile Ala Leu Lys Asp Glu Thr Asn Pro Ile Glu Glu Trp Gln
370 375 380
His Ile Glu Gln Leu Lys Gly Ser Ala Glu Gly Ser Ile Arg Tyr Pro
385 390 395 400
Ala Trp Asn Gly Ile Ile Ser Gln Glu Val Leu Asp Tyr Leu Ser Ser
405 410 415
Tyr Ile Asn Arg Arg Ile
420

<210> 62
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> IR2

<400> 62
gaagttccta ttccgaagtt cctattctct agaaagtata ggaacttc 48

<210> 63
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> IR1

<400> 63
gaagttccta tactttctag agaataggaa cttcggaata ggaacttc 48

<210> 64
<211> 66
<212> DNA
<213> Bacteriophage mu

<220>
<221> CDS
<222> (1)...(66)
<223> nucleotide sequence encoding GIN recombinase

<400> 64
tca act ctg tat aaa aaa cac ccc gcg aaa cga gcg cat ata gaa aac 48
Ser Thr Leu Tyr Lys Lys His Pro Ala Lys Arg Ala His Ile Glu Asn
1 5 10 15

gac gat cga atc aat taa 66
Asp Asp Arg Ile Asn *

20

<210> 65
<211> 21
<212> PRT
<213> bacteriophage mu

<400> 65
Ser Thr Leu Tyr Lys Lys His Pro Ala Lys Arg Ala His Ile Glu Asn
1 5 10 15
Asp Asp Arg Ile Asn
20

<210> 66
<211> 69
<212> DNA
<213> Bacteriophage mu

<220>
<221> CDS
<222> (1)...(69)
<223> nucleotide sequence encoding Gin recombinase

<400> 66
tat aaa aaa cat ccc gcg aaa cga acg cat ata gaa aac gac gat cga 48
Tyr Lys Lys His Pro Ala Lys Arg Thr His Ile Glu Asn Asp Asp Arg
1 5 10 15

atc aat caa atc gat cgg taa 69
Ile Asn Gln Ile Asp Arg *
20

<210> 67
<211> 22
<212> PRT
<213> bacteriophage mu

<220>
<223> Gin recombinase of bacteriophage mu

<400> 67
Tyr Lys Lys His Pro Ala Lys Arg Thr His Ile Glu Asn Asp Asp Arg
1 5 10 15
Ile Asn Gln Ile Asp Arg
20

<210> 68
<211> 555
<212> DNA
<213> Escherichia coli

<220>
<221> CDS
<222> (1)...(555)
<223> nucleotide sequence encoding PIN recombinase

<400> 68
atg ctt att ggc tat gta cgc gta tca aca aat gac cag aac aca gat 48
Met Leu Ile Gly Tyr Val Arg Val Ser Thr Asn Asp Gln Asn Thr Asp
1 5 10 15

cta caa cgt aat gcg ctg aac tgt gca gga tgc gag ctg att ttt gaa 96
Leu Gln Arg Asn Ala Leu Asn Cys Ala Gly Cys Glu Leu Ile Phe Glu
20 25 30

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gac aag ata agc ggc aca aag tcc gaa agg ccg gga ctg aaa aaa ctg       144
Asp Lys Ile Ser Gly Thr Lys Ser Glu Arg Pro Gly Leu Lys Lys Leu
         35                  40                  45

ctc agg aca tta tcg gca ggt gac act ctg gtt gtc tgg aag ctg gat       192
Leu Arg Thr Leu Ser Ala Gly Asp Thr Leu Val Val Trp Lys Leu Asp
     50                  55                  60

cgg ctg ggg cgt agt atg cgg cat ctt gtc gtg ctg gtg gag gag ttg       240
Arg Leu Gly Arg Ser Met Arg His Leu Val Val Leu Val Glu Glu Leu
 65                  70                  75                  80

cgc gaa cga ggc atc aac ttt cgt agt ctg acg gat tca att gat acc       288
Arg Glu Arg Gly Ile Asn Phe Arg Ser Leu Thr Asp Ser Ile Asp Thr
                 85                  90                  95

agc aca cca atg gga cgc ttt ttc ttt cat gtg atg ggt gcc ctg gct       336
Ser Thr Pro Met Gly Arg Phe Phe Phe His Val Met Gly Ala Leu Ala
            100                 105                 110

gaa atg gag cgt gaa ctg att gtt gaa cga aca aaa gct gga ctg gaa       384
Glu Met Glu Arg Glu Leu Ile Val Glu Arg Thr Lys Ala Gly Leu Glu
        115                 120                 125

act gct cgt gca cag gga cga att ggt gga cgt cgt ccc aaa ctt aca       432
Thr Ala Arg Ala Gln Gly Arg Ile Gly Gly Arg Arg Pro Lys Leu Thr
    130                 135                 140

cca gaa caa tgg gca caa gct gga cga tta att gca gca gga act cct       480
Pro Glu Gln Trp Ala Gln Ala Gly Arg Leu Ile Ala Ala Gly Thr Pro
145                 150                 155                 160

cgc cag aag gtg gcg att atc tat gat gtt ggt gtg tca act ttg tat       528
Arg Gln Lys Val Ala Ile Ile Tyr Asp Val Gly Val Ser Thr Leu Tyr
                165                 170                 175

aag agg ttt cct gca ggg gat aaa taa                                   555
Lys Arg Phe Pro Ala Gly Asp Lys  *
            180
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<210> 69
<211> 184
<212> PRT
<213> Escherichia coli

<400> 69
Met Leu Ile Gly Tyr Val Arg Val Ser Thr Asn Asp Gln Asn Thr Asp
 1               5                  10                  15
Leu Gln Arg Asn Ala Leu Asn Cys Ala Gly Cys Glu Leu Ile Phe Glu
            20                  25                  30
Asp Lys Ile Ser Gly Thr Lys Ser Glu Arg Pro Gly Leu Lys Lys Leu
        35                  40                  45
Leu Arg Thr Leu Ser Ala Gly Asp Thr Leu Val Val Trp Lys Leu Asp
    50                  55                  60
Arg Leu Gly Arg Ser Met Arg His Leu Val Val Leu Val Glu Glu Leu
65                  70                  75                  80
Arg Glu Arg Gly Ile Asn Phe Arg Ser Leu Thr Asp Ser Ile Asp Thr
                85                  90                  95
Ser Thr Pro Met Gly Arg Phe Phe Phe His Val Met Gly Ala Leu Ala
            100                 105                 110
Glu Met Glu Arg Glu Leu Ile Val Glu Arg Thr Lys Ala Gly Leu Glu
        115                 120                 125
Thr Ala Arg Ala Gln Gly Arg Ile Gly Gly Arg Arg Pro Lys Leu Thr
    130                 135                 140
Pro Glu Gln Trp Ala Gln Ala Gly Arg Leu Ile Ala Ala Gly Thr Pro
145                 150                 155                 160
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Arg Gln Lys Val Ala Ile Ile Tyr Asp Val Gly Val Ser Thr Leu Tyr
165 170 175
Lys Arg Phe Pro Ala Gly Asp Lys
180

<210> 70
<211> 4778
<212> DNA
<213> Artificial Sequence

<220>
<223> pcx plasmid

<400> 70
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ccaacgaccc ccgcccattg acgtcaataa tgacgtatgt tcccatagta acgccaatag 180
ggactttcca ttgacgtcaa tgggtggact atttacggta aactgcccac ttggcagtac 240
atcaagtgta tcatatgcca agtacgcccc ctattgacgt caatgacggt aaatggcccg 300
cctggcatta tgcccagtac atgaccttat gggactttcc tacttggcag tacatctacg 360
tattagtcat cgctattacc atgggtcgag gtgagcccca cgttctgctt cactctcccc 420
atctcccccc cctccccacc cccaattttg tatttattta ttttttaatt attttgtgca 480
gcgatggggg cggggggggg gggggcgcgc gccaggcggg gcggggcggg gcgaggggcg 540
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tccttttatg gcgaggcggc ggcggcggcg gccctataaa aagcgaagcg cgcggcgggc 660
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ttttccttac atgttttact agccagattt ttcctcctct cctgactact cccagtcata 2220
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gttgcgctca ctgcccgctt tccagtcggg aaacctgtcg tgccagcgga tccgcatctc 2460
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agttccgccc attctccgcc ccatggctga ctaatttttt ttatttatgc agaggccgag 2580
gccgcctcgg cctctgagct attccagaag tagtgaggag gcttttttgg aggcctaggc 2640
ttttgcaaaa agctaacttg tttattgcag cttataatgg ttacaaataa agcaatagca 2700
tcacaaattt cacaaataaa gcattttttt cactgcattc tagttgtggt ttgtccaaac 2760
tcatcaatgt atcttatcat gtctggatcc gctgcattaa tgaatcggcc aacgcgcggg 2820
gagaggcggt ttgcgtattg ggcgctcttc cgcttcctcg ctcactgact cgctgcgctc 2880
ggtcgttcgg ctgcggcgag cggtatcagc tcactcaaag gcggtaatac ggttatccac 2940
agaatcaggg gataacgcag gaaagaacat gtgagcaaaa ggccagcaaa aggccaggaa 3000
ccgtaaaaag gccgcgttgc tggcgttttt ccataggctc cgcccccctg acgagcatca 3060
caaaaatcga cgctcaagtc agaggtggcg aaacccgaca ggactataaa gataccaggc 3120
gtttccccct ggaagctccc tcgtgcgctc tcctgttccg accctgccgc ttaccggata 3180

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cctgtccgcc tttctccctt cgggaagcgt ggcgctttct caatgctcac gctgtaggta   3240
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gcccgaccgc tgcgccttat ccggtaacta tcgtcttgag tccaacccgg taagacacga   3360
cttatcgcca ctggcagcag ccactggtaa caggattagc agagcgaggt atgtaggcgg   3420
tgctacagag ttcttgaagt ggtggcctaa ctacggctac actagaagga cagtatttgg   3480
tatctgcgct ctgctgaagc cagttacctt cggaaaaaga gttggtagct cttgatccgg   3540
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<210> 103
<211> 27
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<220>
<223> MSAT-Ri Primer

<400> 103
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<210> 104
<211> 277
<212> DNA
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<220>
<223> Nopaline Synthase Promoter Sequence

<300>
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aattaacatg taatgcatga cgttatttat gagatgggtt tttatgatta gagtcccgca 180
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gcgcgcggtg tcatctatgt tactagatcg ggaattc 277

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<222> (1)...(1812)
<223> Beta-Glucuronidase

<300>
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<309> 1994-09-23

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Gly Leu Trp Ala Phe Ser Leu Asp Arg Glu Asn Cys Gly Ile Asp Gln
20 25 30

cgt tgg tgg gaa agc gcg tta caa gaa agc cgg gca att gct gtg cca 144
Arg Trp Trp Glu Ser Ala Leu Gln Glu Ser Arg Ala Ile Ala Val Pro
35 40 45

ggc agt ttt aac gat cag ttc gcc gat gca gat att cgt aat tat gcg 192
Gly Ser Phe Asn Asp Gln Phe Ala Asp Ala Asp Ile Arg Asn Tyr Ala
50 55 60

ggc aac gtc tgg tat cag cgc gaa gtc ttt ata ccg aaa ggt tgg gca 240
Gly Asn Val Trp Tyr Gln Arg Glu Val Phe Ile Pro Lys Gly Trp Ala
65 70 75 80

ggc cag cgt atc gtg ctg cgt ttc gat gcg gtc act cat tac ggc aaa 288
Gly Gln Arg Ile Val Leu Arg Phe Asp Ala Val Thr His Tyr Gly Lys
85 90 95

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Val Trp Val Asn Asn Gln Glu Val Met Glu His Gln Gly Gly Tyr Thr
100 105 110

cca ttt gaa gcc gat gtc acg ccg tat gtt att gcc ggg aaa agt gta 384
Pro Phe Glu Ala Asp Val Thr Pro Tyr Val Ile Ala Gly Lys Ser Val
115 120 125

cgt atc acc gtt tgt gtg aac aac gaa ctg aac tgg cag act atc ccg 432
Arg Ile Thr Val Cys Val Asn Asn Glu Leu Asn Trp Gln Thr Ile Pro
130 135 140

ccg gga atg gtg att acc gac gaa aac ggc aag aaa aag cag tct tac 480
Pro Gly Met Val Ile Thr Asp Glu Asn Gly Lys Lys Lys Gln Ser Tyr
145 150 155 160

ttc cat gat ttc ttt aac tat gcc gga atc cat cgc agc gta atg ctc 528
Phe His Asp Phe Phe Asn Tyr Ala Gly Ile His Arg Ser Val Met Leu
165 170 175

tac acc acg ccg aac acc tgg gtg gac gat atc acc gtg gtg acg cat 576

Tyr Thr Thr Pro Asn Thr Trp Val Asp Asp Ile Thr Val Val Thr His
180 185 190

gtc gcg caa gac tgt aac cac gcg tct gtt gac tgg cag gtg gtg gcc 624
Val Ala Gln Asp Cys Asn His Ala Ser Val Asp Trp Gln Val Val Ala
195 200 205

aat ggt gat gtc agc gtt gaa ctg cgt gat gcg gat caa cag gtg gtt 672
Asn Gly Asp Val Ser Val Glu Leu Arg Asp Ala Asp Gln Gln Val Val
210 215 220

gca act gga caa ggc act agc ggg act ttg caa gtg gtg aat ccg cac 720
Ala Thr Gly Gln Gly Thr Ser Gly Thr Leu Gln Val Val Asn Pro His
225 230 235 240

ctc tgg caa ccg ggt gaa ggt tat ctc tat gaa ctg tgc gtc aca gcc 768
Leu Trp Gln Pro Gly Glu Gly Tyr Leu Tyr Glu Leu Cys Val Thr Ala
245 250 255

aaa agc cag aca gag tgt gat atc tac ccg ctt cgc gtc ggc atc cgg 816
Lys Ser Gln Thr Glu Cys Asp Ile Tyr Pro Leu Arg Val Gly Ile Arg
260 265 270

tca gtg gca gtg aag ggc gaa cag ttc ctg att aac cac aaa ccg ttc 864
Ser Val Ala Val Lys Gly Glu Gln Phe Leu Ile Asn His Lys Pro Phe
275 280 285

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Tyr Phe Thr Gly Phe Gly Arg His Glu Asp Ala Asp Leu Arg Gly Lys
290 295 300

gga ttc gat aac gtg ctg atg gtg cac gac cac gca tta atg gac tgg 960
Gly Phe Asp Asn Val Leu Met Val His Asp His Ala Leu Met Asp Trp
305 310 315 320

att ggg gcc aac tcc tac cgt acc tcg cat tac cct tac gct gaa gag 1008
Ile Gly Ala Asn Ser Tyr Arg Thr Ser His Tyr Pro Tyr Ala Glu Glu
325 330 335

atg ctc gac tgg gca gat gaa cat ggc atc gtg gtg att gat gaa act 1056
Met Leu Asp Trp Ala Asp Glu His Gly Ile Val Val Ile Asp Glu Thr
340 345 350

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Ala Ala Val Gly Phe Asn Leu Ser Leu Gly Ile Gly Phe Glu Ala Gly
355 360 365

aac aag ccg aaa gaa ctg tac agc gaa gag gca gtc aac ggg gaa act 1152
Asn Lys Pro Lys Glu Leu Tyr Ser Glu Glu Ala Val Asn Gly Glu Thr
370 375 380

cag caa gcg cac tta cag gcg att aaa gag ctg ata gcg cgt gac aaa 1200
Gln Gln Ala His Leu Gln Ala Ile Lys Glu Leu Ile Ala Arg Asp Lys
385 390 395 400

aac cac cca agc gtg gtg atg tgg agt att gcc aac gaa ccg gat acc 1248
Asn His Pro Ser Val Val Met Trp Ser Ile Ala Asn Glu Pro Asp Thr
405 410 415

cgt ccg caa ggt gca cgg gaa tat ttc gcg cca ctg gcg gaa gca acg 1296
Arg Pro Gln Gly Ala Arg Glu Tyr Phe Ala Pro Leu Ala Glu Ala Thr
420 425 430

cgt aaa ctc gac ccg acg cgt ccg atc acc tgc gtc aat gta atg ttc 1344
Arg Lys Leu Asp Pro Thr Arg Pro Ile Thr Cys Val Asn Val Met Phe
435 440 445

tgc gac gct cac acc gat acc atc agc gat ctc ttt gat gtg ctg tgc 1392
Cys Asp Ala His Thr Asp Thr Ile Ser Asp Leu Phe Asp Val Leu Cys
450 455 460

ctg aac cgt tat tac gga tgg tat gtc caa agc ggc gat ttg gaa acg 1440
Leu Asn Arg Tyr Tyr Gly Trp Tyr Val Gln Ser Gly Asp Leu Glu Thr
465 470 475 480

gca gag aag gta ctg gaa aaa gaa ctt ctg gcc tgg cag gag aaa ctg 1488
Ala Glu Lys Val Leu Glu Lys Glu Leu Leu Ala Trp Gln Glu Lys Leu
485 490 495

cat cag ccg att atc atc acc gaa tac ggc gtg gat acg tta gcc ggg 1536
His Gln Pro Ile Ile Ile Thr Glu Tyr Gly Val Asp Thr Leu Ala Gly
500 505 510

ctg cac tca atg tac acc gac atg tgg agt gaa gag tat cag tgt gca 1584
Leu His Ser Met Tyr Thr Asp Met Trp Ser Glu Glu Tyr Gln Cys Ala
515 520 525

tgg ctg gat atg tat cac cgc gtc ttt gat cgc gtc agc gcc gtc gtc 1632
Trp Leu Asp Met Tyr His Arg Val Phe Asp Arg Val Ser Ala Val Val
530 535 540

ggt gaa cag gta tgg aat ttc gcc gat ttt gcg acc tcg caa ggc ata 1680
Gly Glu Gln Val Trp Asn Phe Ala Asp Phe Ala Thr Ser Gln Gly Ile
545 550 555 560

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Leu Arg Val Gly Gly Asn Lys Lys Gly Ile Phe Thr Arg Asp Arg Lys
565 570 575

ccg aag tcg gcg gct ttt ctg ctg caa aaa cgc tgg act ggc atg aac 1776
Pro Lys Ser Ala Ala Phe Leu Leu Gln Lys Arg Trp Thr Gly Met Asn
580 585 590

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<213> Escherichia coli

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<309> 1994-09-23

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35 40 45
Gly Ser Phe Asn Asp Gln Phe Ala Asp Ala Asp Ile Arg Asn Tyr Ala
50 55 60
Gly Asn Val Trp Tyr Gln Arg Glu Val Phe Ile Pro Lys Gly Trp Ala
65 70 75 80
Gly Gln Arg Ile Val Leu Arg Phe Asp Ala Val Thr His Tyr Gly Lys
85 90 95
Val Trp Val Asn Asn Gln Glu Val Met Glu His Gln Gly Gly Tyr Thr
100 105 110
Pro Phe Glu Ala Asp Val Thr Pro Tyr Val Ile Ala Gly Lys Ser Val
115 120 125

Arg Ile Thr Val Cys Val Asn Asn Glu Leu Asn Trp Gln Thr Ile Pro
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Pro Gly Met Val Ile Thr Asp Glu Asn Gly Lys Lys Lys Gln Ser Tyr
145   150   155   160
Phe His Asp Phe Phe Asn Tyr Ala Gly Ile His Arg Ser Val Met Leu
                165   170   175
Tyr Thr Thr Pro Asn Thr Trp Val Asp Asp Ile Thr Val Val Thr His
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Val Ala Gln Asp Cys Asn His Ala Ser Val Asp Trp Gln Val Val Ala
        195   200   205
Asn Gly Asp Val Ser Val Glu Leu Arg Asp Ala Asp Gln Gln Val Val
    210   215   220
Ala Thr Gly Gln Gly Thr Ser Gly Thr Leu Gln Val Val Asn Pro His
225   230   235   240
Leu Trp Gln Pro Gly Glu Gly Tyr Leu Tyr Glu Leu Cys Val Thr Ala
                245   250   255
Lys Ser Gln Thr Glu Cys Asp Ile Tyr Pro Leu Arg Val Gly Ile Arg
            260   265   270
Ser Val Ala Val Lys Gly Glu Gln Phe Leu Ile Asn His Lys Pro Phe
        275   280   285
Tyr Phe Thr Gly Phe Gly Arg His Glu Asp Ala Asp Leu Arg Gly Lys
    290   295   300
Gly Phe Asp Asn Val Leu Met Val His Asp His Ala Leu Met Asp Trp
305   310   315   320
Ile Gly Ala Asn Ser Tyr Arg Thr Ser His Tyr Pro Tyr Ala Glu Glu
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Met Leu Asp Trp Ala Asp Glu His Gly Ile Val Val Ile Asp Glu Thr
            340   345   350
Ala Ala Val Gly Phe Asn Leu Ser Leu Gly Ile Gly Phe Glu Ala Gly
        355   360   365
Asn Lys Pro Lys Glu Leu Tyr Ser Glu Glu Ala Val Asn Gly Glu Thr
    370   375   380
Gln Gln Ala His Leu Gln Ala Ile Lys Glu Leu Ile Ala Arg Asp Lys
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Arg Pro Gln Gly Ala Arg Glu Tyr Phe Ala Pro Leu Ala Glu Ala Thr
            420   425   430
Arg Lys Leu Asp Pro Thr Arg Pro Ile Thr Cys Val Asn Val Met Phe
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Cys Asp Ala His Thr Asp Thr Ile Ser Asp Leu Phe Asp Val Leu Cys
    450   455   460
Leu Asn Arg Tyr Tyr Gly Trp Tyr Val Gln Ser Gly Asp Leu Glu Thr
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Ala Glu Lys Val Leu Glu Lys Glu Leu Leu Ala Trp Gln Glu Lys Leu
                485   490   495
His Gln Pro Ile Ile Ile Thr Glu Tyr Gly Val Asp Thr Leu Ala Gly
            500   505   510
Leu His Ser Met Tyr Thr Asp Met Trp Ser Glu Glu Tyr Gln Cys Ala
        515   520   525
Trp Leu Asp Met Tyr His Arg Val Phe Asp Arg Val Ser Ala Val Val
    530   535   540
Gly Glu Gln Val Trp Asn Phe Ala Asp Phe Ala Thr Ser Gln Gly Ile
545   550   555   560
Leu Arg Val Gly Gly Asn Lys Lys Gly Ile Phe Thr Arg Asp Arg Lys
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<220>
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<300>
<308> U09365
<309> 1995-10-17

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the Mast1 sequence

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/17452

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C07H 21/04; C12N 15/85,87,90; A01N 43/34; C12N 15/09
US CL : 536/23.1; 435/320.1,325,419,455,467; 514/44; 800/21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 536/23.1; 435/320.1,325,419,455,467; 514/44; 800/21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS (EAST); STN (MEDLINE, BIOSIS, CAPLUS)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 01/07572 A2 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 01 February 2001 (01.02.2001), see entire document.	1,2,7,8,11,23-25,27,50 ---------- 3-6,9,10,12,13,26,28-
X --- Y	WO 00/11155 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 02March 2000 (02.03.2000), see entire document.	1,2,7,8,11,23-25,27,50 ---------- 3-6,9,10,12,13,26,28-
X --- Y	US 6,171,861 B1 (HARTLEY et al.) 09 January 2001 (09.01.2001), see entire document, especially sequence listing.	1-3,8,10-12,23-27,33,36,39,40,43-45,50,54,55,85 ---------- 4-7,9,13-22,28-30,32,34,35,37,38,41,42,46-49,51-53,56,67-71,84,86

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
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INTERNATIONAL SEARCH REPORT

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 96/40724 A1 (LIFE TECHNOLOGIES, INC.) 19 December 1996 (19.12.96), see entire document, especially SEQ ID NO:1-16.	1-3,8,10-12,23-27,33,36,39,40,43-45,50,54,55,85 --- 1-3,8,10-12,23-27,33,36,39,40,43-45,50,54,55,85
Y	WO 94/00569 A1 (GENPHARM INTERNATIONAL, INC.) 06 January 1994 (06.01.94), see entire document.	72-78
Y	WO 94/23049 A2 (THE JOHNS HOPKINS UNIVERSITY) 13 October 1994 (13.10.94), see entire document.	72-78
X --- Y	US 5,721,118 A (SCHEFFLER) 24 February 1998 (24.02.98), see entire document, especially Example 3.	14,15,22-25,57 --- 16-21,26,58-64,72-78,84,85,98-105,123
X --- Y	US 5,948,653 A (PATI et al.) 07 September 1999 (07.09.99), see entire document, especially the first full paragraph in column 29.	14,15,22-25,57 --- 16-21,26,58-64,72-78,84,85,98-105,123
X --- Y	GB 2 331 752 A (MEDICAL RESEARCH COUNCIL) 02 June 1999 (02.06.99), see entire document, especially pages 8-10.	109,123 --- 72-78,110-122
A	HALDIMANN et al. Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene strucure-function studies of bacteria. J Bacteriol. November 2001, Vol. 183 No. 21, pages 6384-6393, see entire document.	65,66
X,P --- Y,P	MORALLI et al. Insertion of a loxP site in a size-reduced human accessory chromosome. Cytogenet. Cell Genet. 2001, Vol. 94 No. 3-4, pages 113-120, see entire document.	91-93,106,108 --- 94-97,107
X --- Y	LORBACH et al. Site-specific recombination in human cells catalyzed by phage lambda integrase mutants. J. Mol. Biol. March 2000, Vol. 296 No. 5, pages 1175-1181, see entire document.	1,2,8,50 --- 3-7,9-13,23-32,51-56,84,85
X --- Y	CALL et al. A cre-lox recombination system for the targeted integration of circular yeast artificial chromosomes into embryonic stem cells.Hum. Mol. Genet. July 2000, Vol. 9, No. 12, pages 1745-1751, see entire document.	14,15,22-26,57,59 --- 16-21,58,60,84,85,98-105
Y,P	HADLACZKY, G. Satellite DNA-based artificial chromosomes for use in gene therapy. Curr. Opin. Mol. Ther. April 2001, Vol. 3 No. 2, pages 125-132, see entire document.	1-123

INTERNATIONAL SEARCH REPORT

PCT/US02/17452

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-64, 67-71, 79, 84-86, 91-108, and 123, drawn to eukaryotic recombinogenic chromosomes used for introducing heterologous nucleic acids into a chromosome and the resulting cells.
Group II, claim(s) 65-66 and 87-88, drawn to a lambda intR mutein.
Group III, claim(s) 72-78, drawn to the production of transgenic animals.
Group IV, claim(s) 80, drawn to the production of an artificial chromosome library.
Group V, claim(s) 81-83, drawn to a library of cells for genomic screening.
Group VI, claim(s) 89-90, drawn to a modified iron-induced promoter.
Group VII, claim(s) 109-122, drawn to a method for screening compounds and their effects on regulatory regions.

and it considers that the International Application does not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated below:

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of Group I which defines an advance over the art is a eukaryotic chromosome containing recombinogenic sites that can be used to introduce heterologous nucleic acids into chromosomes, and cells containing these recombinogenic chromosomes.
The special technical feature of Group II involves a lambda intR mutein. This feature defines an advance over Group I in that it involves a protein that is not required for the technical feaures as set forth above in Group I.
The special technical feature of Group III involves the production of a transgenic animal, which represents a second method for using the invention as set forth above in Group I.
The special technical feature of Group IV involves the production of artificial chromosome expression system libraries, which represents a third method for using the invention as set forth above in Group I.
The special technical feature of Group V involves a library of cells containing the artificial chromosome expression system libraries set foth above in Group IV, and represents a first product resulting from Group IV.
The special technical feature of Group VI involves a modified iron-inducible promoter. This feature defines an advance over Group I in that it involves a promoter that is not required for the technical features as set forth above in Group I.
The special technical feature of Group VII involves a method for screening compounds for their effect on regulatory regions, which represents a fourth method of using the invention as set forth above in Group I.

INTERNATIONAL SEARCH REPORT	International application No. PCT/US02/17452

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.